Medical Microbiology and Infectious Diseases:-

A STUDY

OF

CRYPTOCOCCUS NEOFORMANS

VARIETIES

GATTII AND *NEOFORMANS*

IAN F. LAURENSON

Submitted for the degree of Doctor of Medicine

University of Edinburgh

1997-8

ABSTRACT

Cryptococcus neoformans is a fungus that can be biotyped as variety (var.) *gattii* or var. *neoformans.* Four primary serotypes, A,B,C, and D have been described. *Cryptococcus neoformans* var. *gattii* is predominant in the tropics and subtropics.

Environmental investigations have established the association of *Cryptococcus neoformans* var. *gattii* with *Eucalyptus camaldulensis*, *E. tereticornis*, *E. gomphcephala*, *E. rudis* and most recently *E. blaykeli.* Worldwide, *C. neoformans* var. *neoformans* has been associated with avian sources. This thesis examines *C. neoformans* and the meningitis it causes, based predominantly in Papua New Guinea (PNG).

Investigations of possible plant, mammal and avian associations in PNG, revealed that few *E. camaldulensis* survive experimental planting, while *E. tereticornis* is endemic. In Port Moresby, *E. confertiflora*, *E. papuana* and *E. alba* are common. Examination of 1130 specimens from plant, bird and animal sources, failed to identify the ecological niche of *C. neoformans* in PNG.

Epidemiological studies of 96 patients presenting with cryptococcal meningitis to Port Moresby General Hospital (PMGH) from 1972-1993 showed an annual incidence of 33 cases per million population of Central Province and the National Capital District. Twenty one of these are infected with *C. neoformans* var. *gattii* and 12 with *C. neoformans* var. *neoformans.* On average 11 cases present annually

to PMGH. Geographical clustering occurred amongst those from some parts of Gulf and Central Provinces, with a male predominance. Possible seasonal variation was found, with increased presentation rates in May/June and September/October. This could reflect seasonal exposure to *Cryptococcus neoformans* var. *gattii.* The pattern of childhood infection may result from varying exposure or susceptibility.

Eleven sequential patients with cryptococcal meningitis were diagnosed and isolates biotyped. Seven were var. *gattii* (one patient with diabetes mellitus) and four were var. *neoformans.* The latter came from adult patients with HIV 1 infection, tuberculosis or *Plasmodium vivax* malaria. Significant clinical findings were headache, fever, meningism, vomiting, photophobia, papilloedema and cranial nerve lesions. Five patients (45.5%) died; the 2 var. *neoformans* HIV 1 infected men and 3 adult var. *gattii* patients. In PNG where var. *gattii* has been predominant in the immunocompetent, var. *neoformans* is emerging as the predominant biotype amongst immunosuppressed patients, notably those with HIV1 infection.

Laboratory comparison in Edinburgh confirmed that var. *gattii* were usually more mucoid than var. *neoformans* colonies and that this was correlated with capsule size. A neutrophil-monolayer assay was unable to demonstrate a clear difference in varietal binding.

Cerebrospinal fluid examination by microscopy and cryptococcal latex antigen failed to detect an apparently acapsulate cryptococcal isolate in an HIV infected

patient in Edinburgh. The isolate was detected by culture on malt extract agar. The human neutrophil-cryptococcal assay subsequently confirmed the presence of capsule.

These studies confirm the high prevalence in PNG of meningitis caused by *C. neoformans* variety *gattii* in immunocompetent individuals, and the presence of potential mammal and plant sources similar to those found in Australia. Although cases are clustered geographically, the source(s) of *C. neoformans* remain elusive. Alone, the small series of prospectively studied patients cannot indicate whether the course of meningitis caused by the two varieties of *C. neoformans* differs in the immunocompetent. Laboratory studies investigated the intriguing differences in pathogenesis between the two varieties and emphasise the importance of culture in clinical diagnosis.

The work described here has formed a foundation for further clinical, epidemiological and laboratory studies of *C. neoformans* meningitis in PNG and London, England.

FORMAL DECLARATION

I declare that this thesis has been composed by myself and that I have carried out the work itself except where indicated.

I.F.Laurenson

Dated: 21st June 1998

Acknowledgements

There are many friends and colleagues who have taken an interest in and helped with these studies. I would like to mention:

Professor David Warrell, David Lalloo, Andrew Trevett and Julie Black for getting me to Papua New Guinea, and their continued support and friendship during and after my time there.

Professor Sirus Naraqi, Professor Isi Kevau, Adolf Saweri, Barnabas Mavo, Laboratory Staff, friends and colleagues at Port Morseby General Hospital, for encouraging and allowing me to pursue this particular interest, and for making me so welcome. Tessi the hospital social worker helped greatly in long term follow up of patients. Andrew Seaton and Asi Matuka helped to complete the environmental sampling studies.

David Ellis and Tania Pfeiffer in Adelaide, and Professor DWR Mackenzie and Colin Campbell in London offered important mycological support, albeit from afar.

Max Kuduk, Neville Howcroft, Ian Burrows and Simon Saulei gave me a thoughtful and helpful introduction to the flora and fauna of Papua New Guinea.

David Baker, Bruce Harris and Malcolm Baldock supported me in computer technology and scanning of photographs.

Professor JD Williams allowed me to take a 'sabbatical' period away from my Microbiology post in London.

Rex Miles and John Peutherer, Professor Sebastian Amyes, friends and colleagues who have given me the opportunity to continue studies in the Department of Medical Microbiology, University of Edinburgh. This included bench space and a Departmental Grant.

Malcolm Richardson and Mandy Wiggins enthusiastically encouraged me and advised on my Edinburgh based cryptococcal laboratory studies. John Stewart has also advised on cryptococcal-neutrophil studies and Mike Kerr given technical assistance in these. Stephanie Chisolm also helped with some initial experiments.

Emma my wife has encouraged and supported me in completing this thesis, proof reading it and preparing good suppers!

Finally and importantly I thank my Parents for "giving me roots and giving me wings".

Contents

List of Tables

List of Figures

List of Plates

Publications

1.
Laurenson, I.F, Naraqi, S., Howcroft, N., Burrows, I. and Saulei, S. (1993) Cryptococcal meningitis in Papua New Guinea: ecology and the role of eucalypts. *Med J Aust* **158**, 213.

2.
Lalloo, D., Fisher, D., Naraqi, S., **Laurenson, I.**, Temu, P., Sinha, A., Saweri, A. and Mavo, B. (1994) Cryptococcal meningitis (*C. neoformans* var. *gattii)* leading to blindness in previously healthy Melanesian adults in Papua New Guinea. *Q J Med* **87**, 343-349.

3.
Laurenson, I.F., Trevett, A.J., Lalloo, D.G., Nwokolo, N., Naraqi, S., Black, J., Tefurani, N., Saweri, A., Mavo, B., Igo, J. and Warrell, D.A. (1996) Meningitis caused by *Cryptococcus neoformans* varieties *gattii* and *neoformans* in Papua New Guinea. *Trans R Soc Trop Med Hyg* **90**, 57-60.

4.
Laurenson I.F. (1997) "Cosmopolitan cryptococcal meningoencephalitis". *Rev Med Microbiol* **8,** 41-50

5.
Laurenson, I.F., Lalloo, D.G., Naraqi, S., Seaton, R.A., Trevett, A.J., Matuka, A. and Kevau, I.H. (1997) *Cryptococcus neoformans* in Papua New Guinea; a common pathogen but an elusive source. *J Med Vet Mycol* **35** 437-440.

6.
Laurenson, I.F., Ross, J.D.C., Milne, L.J.R. (1998) Microscopy and latex antigen negative cryptococcal meningitis. *J Infect* **36** 329-331.

Conference Abstracts

1.
Laurenson I.F., Naraqi S., Lalloo D., Trevett A., Saweri A. "Developments in Cryptococcal Meningitis in Papua New Guinea". Australian Society for Microbiology & New Zealand Microbiology Society Combined Scientific Meeting, Sydney, July 1992

2.
Laurenson I.F., Naraqi S. Lalloo D., Igo J. "Cryptococcal Meningitis in Papua New Guinea" XIIIth International Congress for Tropical Medicine & Malaria, Jontiem, Thailand, December 1992

3.
Laurenson I.F., Naraqi S., Lalloo D.,Trevett A., Nwokolo N., Ogunbanjo B., Igo J.,Saweri A., Tefurani N. "Cryptococcal Investigations in Papua New Guinea" 2nd International Conference on *Cryptococcus* and Cryptococcosis, Milan, September, 1993.

4.
Laurenson I.F., Chisolm SJ, Kerr M, Stewart J. "Cryptococcal-human neutrophil interactions" 3rd International Conference on *Cryptococcus* and Cryptococcosis, Paris, September 1996.

Coauthors' contributions to publications:

1. Coauthors provided advice on flora and fauna of Papua New Guinea and access to Herbariums.

2. Coauthors allowed access to, assembled case notes and initiated this study. My main roles were in following up patients and analysing survival data as well as contributing to drafts of the text.

3. I organised and undertook this study under the supervision of SN and DAW. Coauthors allowed access to their patients and the hospital laboratory. AJT, DGL and NN helped in data collection principally if I was on leave.

4. I prepared and wrote this article alone.

5. I organised and carried out this study under the supervision of SN and continued later under IHK. Later samples were processed by Mr. A.Matuka under the supervision of AJT and RAS who also collected some samples. DGL helped with discussion and a few collections.
6. I helped diagnose the case, formulated the case report and wrote it up with input from JDC and LJRM.

Coauthors' contibutions to conference abstracts:

1-3. As in publications above.

4. I formulated and organised these studies and carried out experimental work with supervision from JS. MJK and SJC provided additional technical input.

Unpublished work

All other work described in this thesis was performed by myself. Any additional assistance is identified in the Acknowledgements.

Chapter 1

Introduction

1.1 Introduction

1.1.1 History

There are 19 known species of *Cryptococcus* (Rippon, 1988), but the aetiological agent in virtually all cases of cryptococcosis is *Cryptococcus neoformans*. Just over one hundred years ago it was first described in nature, being isolated from peach juice in Sardinia by Sanfelice in 1894 (Sanfelice, 1894) and described in a tibial lesion of a woman in Germany by Busse and Buschke (Busse, 1894; Buschke, 1894). Sanfelice confirmed its pathogenicity in animal experiments, calling it *Saccharomyces* (Vuillemin, 1901). Sanfelice isolated a similar yeast from the lymph node of an ox lymph with primary liver carcinoma (Sanfelice, 1895) and then in 1902 in Massachusetts, Frothingham identified such a yeast in a pulmonary lesion of a horse (Frothingham, 1902). These findings together confirmed this yeast as the cause of both human and animal disease.

Vuillemin reclassified the fungi identified by Busse and Sanfelice to the genus *Cryptococcus* as *C. hominis* and *C. neoformans* as they neither fermented carbon nor formed ascospores, unlike the *Saccharomyces* genus (Vuillemin, 1901). In 1905 Von Hansemann described a case of meningitis with gelatinous cysts present (von Hanseman, 1905) and in the same year Stoddard and Cutler recorded two cases, interpreting the surrounding capsule as an area of tissue destroyed by the histolytic action of the fungus and naming the causative organism *Torula histolytica* (Stoddard and Cutler, 1916). From this misinterpretation the disease was named torulosis, a term which was used for many years. Nomenclature proved a divisive issue, but this

seems to have been settled in 1950 by Benham (Benham, 1950). Up to that point several names had been used including *Saccharomyces neoformans, Blastomyces neoformans, Cryptococcus hominis* and *Torula histolytica.* Derived from these were a number of terms for cryptococcosis such as torulosis, European blastomycosis and torula meningitis. A new variety based on the observation of an atypical isolate forming elongated cells as well as round cells in mouse brain was described in 1970 (Vanbreuseghem and Takashio, 1970). This variety was called *C. neoformans* variety (var.) *gattii* after its original description by Gattii and Eeckels that year. Taxonomy became further complicated when the sexual, teleomorph, state of *C. neoformans* was described in 1975 in the basideomycemorph genus *Filobasidiella* (Kwon-Chung, 1975). After considerable and complex debate, reviewed by Kwon Chung, she concluded that *Filobasidiella neoformans* var. *neoformans* and *Filobasidiella neoformans* var. *gattii* should be considered as varieties of the same species (Kwon-Chung et al. 1982a). The anamorpic states of these are *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* respectively.

Reviews of reported patients with central nervous system torulosis carried out by Freeman and Levin showed that two thirds of the sixty patients were aged between 30 and 60, with a male to female ratio of two to one (Freeman, 1931; Levin, 1937). Those afflicted were not clustered in place nor had any particular occupation. The onset of illness was slow, occurring over several weeks or months with papilloedema a common finding. Cerebrospinal (CSF) pressure was often raised with lymphocyte predominance and yeasts visible on microscopy which could be cultured on Sabouraud's media. After a variable course all patients died of

respiratory failure after becoming comatose and half had evidence of extrameningeal involvement, particularly in the lung but also in kidneys, spleen, adrenals and skin. Histopathologic examination showed a granulomatous meningitis with cerebral cystic and granulomatous lesions.

1.1.2 Host predisposition

It was highlighted in 1954 that patients with cryptococcosis commonly had an association with malignant disease of the reticuloendothelial system and subsequently other predispositions have been clearly linked such as those with sarcoidosis and corticosteroid therapy (Zimmerman and Rappaport, 1954). These findings were identified in patients predominantly in the United States but in tropical and sub tropical regions this association did not seem to hold true. Cox and Tolhurst reviewed thirteen cases in 1946 that had presented in Australia, dating back to 1914 (Cox and Tolhurst, 1946). They described how four of six autopsied cases had large torulae collections in the brain and five of six had such torulae in the lungs, unlike the findings in the United States series. They also suggested that the incidence was higher than in the United States. A review of experience between 1957 and 1975 of cryptococcosis in the Northern Territory of Australia found that no cases had been treated with corticosteroids or had malignant disease prior to admission (Schupbach et al. 1976). Previously healthy individuals have similarly been predominant amongst those presenting with cryptococcosis elsewhere in Queensland, Australia, Singapore, Malaysia and Zimbabwe (De Wytt et al. 1982; Tjia et al. 1985; Pathmanathan and Soo-Hoo Tuck Soon, 1982; Gould and Gould,

1985). These reports have predated the AIDS epidemic and *C. neoformans* isolates have generally not been biotyped.

1.2 Mycology of *Cryptococcus neoformans*

1.2.1 Introduction

The origins of mycology are botanical, because fungi were initially thought to be part of the plant kingdom and so studied and classified in ways appropriate to plants. 'Mykes' is derived from the Greek for mushrooms. Fungi are absorptive eukaryotes as defined in the Whittaker scheme of classification (Whittaker, 1969). This means that they absorb nutrients from their surroundings, as opposed to using chlorophyll and being photosynthetic (Plantae), or ingestive (Animalia). They have a true nucleus and are characterized by discrete chromosomes bounded by a nuclear membrane, organelles and a cell membrane, typically comprised of ergosterol. Taxonomy is somewhat complex and controversial, at times being complicated by the separate discovery of sexual and asexual forms of the same species. Asexual forms, also known as the anamorphs, reproduce by budding or hyphal extension, whereas the sexual (teleomorph) forms require two mating forms to contribute gametes before reproduction occurs.

1.2.2 *Cryptococcus neoformans*

The teleomorph, sexual form of *C. neoformans* is called *Filobasidiella neoformans*. *C. neoformans* is often referred to as a basidiomycetous yeast-like fungus. The Basidiomycetes are those fungi in which the sexual process involves the production of haploid basidiospores borne on a basidium in which a diploid nucleus undergoes meiosis. Thus for the sexual, teleomorph form *F. neoformans* the sexual propagule is known as a basidiospore. If a fungal isolate does not produce a sexual phase then it is assigned on the more limited information available from asexual sporulation. The form of reproduction by the spores (and their type) dictates the genera into which a fungus is classified. Yeasts, many of which do not spore, have been classified on the basis of the vegatative cell and fermentation tests as well as on the sporulation process. Indeed, in most cases the haploid anamorph (yeast) is isolated from natural or clinical samples.

Over 95% of the fertile natural teleomorph isolates belong to the α mating type of *F. neoformans*. The asexual yeast forms, *C. neoformans*, typically develop a polysaccharide capsule following budding. While the majority of fungi are filamentous with hyphae enabling them to colonise substrata that are nutrient rich, but otherwise difficult to attack, yeasts are fungi that lack or rarely produce hyphae, are unicellular and multiply by budding. Yeasts are abundant in habitats such as the surface of plants and animals. It is likely that yeast forms are superior to hyphae in resisting hydrostatic pressures generated by the fluctuating ambient water potentials or osmotic conditions on such surfaces.

1.2.3 Basidiospore production and the infectious propagule

How often, if at all, sexual reproduction by *F. neoformans* happens in nature is uncertain. The basidiospores are small and dry which would allow them to penetrate the lung to the alveoli, taking on the role of the infectious propagule (Ellis and Pfeiffer, 1990a; Ellis and Pfeiffer, 1992). Clinical isolates *C. neoformans* are generally haploid and virtually always one of the two mating types, the α type (Madrenys et al. 1993; Takeo et al. 1993). During sexual reproduction equal numbers of each mating type are produced. Patients would be expected to have both a and α mating types, but all clinical isolates and most found in nature have the α allele. This information suggests that either the natural infectious propagule is the asexual, yeast form (in this case derived from the α mating type), or that the a mating type does not survive as well in the environment as the α mating type.

Recently Wickes et al showed haploid fruiting of *C. neoformans* in 'starvation conditions' associated with the α mating type locus, independant of serotype (Wickes et al. 1996). Basidiospore production in these circumstances may explain the preponderance of this α mating type in the environment and the nature of the infectious propagule of *C. neoformans*.

1.2.4 Culture

Culture of *Cryptococcus neoformans* on Sabaoraud's dextrose agar or malt extract agar from clinical specimens is optimal at 25-30^{0}C, colonies developing within 36-72 hours. They appear white/cream coloured and have a degree of mucosity reflecting the degree of encapsulation. Microscopy of such colonies shows rounded budding yeasts which are also seen in tissue specimens (Erke and Schneidau, Jr. 1973). Single or multiple buds may be seen which detach easily from the parent form. Examination of a wet preparation gives the impression of a capsule, which is seen more clearly by staining with India ink or phase contrast microscopy.

1.3 Identification, growth and physiology

C. neoformans requires simple carbon and nitrogen sources for growth, but may not need vitamin supplementaion with thiamine (Bruatto et al. 1992). Doubling times at 37^{0}C vary between strains from 2.5 to 6 hours (Miller et al. 1990), which is not as quick as other yeasts such as *Candida albicans* and *Saccharomyces cerevisiae* given similar conditions. The use of low-molecular-weight nitrogenous compounds such as creatinine may partially explain the ecological niche of *C. neoformans* var. *neoformans* in avian guano.

Most other species of *Cryptococcus* are unable to grow at 37^0 C and are not pathogenic whereas *C. neoformans* does grow at this temperature, but is generally killed after 24 hours at 40^0 C. The ability of most strains to secrete urease is used in identification of *C. neoformans* (Zimmer and Roberts, 1979). Another feature is

phenol oxidase production, perhaps acting as an antioxidant so enhancing survival of the yeast in the host (Jacobson et al. 1994; Jacobson and Tinnell, 1993). The presence of this enzyme which results in the production of melanin from catecholamine precursors is used in identification. Pigmented colonies are produced when grown on catechol enriched media such as bird seed agar which can be picked out on plates where other microorganisms are present, for instance in environmental or respiratory specimens (Staib et al. 1987). These features along with resistance to antimicrobial agents such as penicillin, gentamicin and streptomycin are used in isolation and identification from contaminated clinical or environmental specimens (Kwon-Chung and Bennett, 1992; Warren and Hazen, 1995; Staib et al. 1989a). Phenol oxidase activity may account for the predilection of *C. neoformans* for the central nervous system, which is catechol rich, allowing melanin production and protection from host defences (Rhodes et al. 1982).

1.4 Capsule

1.4.1 Serology

Polysaccharide capsular antigen determinants form the basis for serotyping. Initially in 1949 Evans identified differences in rabbit sera following inoculation with different clinical isolates (Evans, 1949). Three serotypes were identified, A, B and C. Almost 20 years later Vogel identified a fourth serotype, D (Vogel, 1966). There are now four recognised serotypes, A,B,C and D. Type-specific antiserum is prepared by immunising rabbits with formalin killed yeast of known serotype, and adsorbing the antiserum with yeast of heterologous serotypes (Wilson et al. 1968). Some capsulate isolates are untypeable on this basis and some react with antisera to

the types A and D and so are called AD (both A and D determinants present). A few isolates are acapsulate and so cannot be typed in this way.

1.4.2 Biochemistry

Capsular polysaccharide from *C. neoformans* grown in vitro or during infection in vivo is soluble and can be precipitated from culture supernatant or detected in bodily fluids such as blood, urine and cerebrospinal fluid (CSF). Culture filtrates contain at least three antigens which have been well defined, glucoronoxylomannan (GXM), galactoxylomannan (GalXM) and a mannoprotein (Murphy et al. 1988).

The main capsule constituent is GXM. Small variations in this constituent, in particular the degree of 0-acetylation of hydroxyl groups and the number of xylose residues, forms the basis for the current serotyping scheme (Turner and Cherniak, 1991; Wilson et al. 1968). The recent application of 13C nuclear resonance spectrophotometry has lead to the realisation that a simple structural relationship between the GXMs of the four serotypes is an oversimplification. Certain structures, considered typical of one serotype have been identified in heterologous serotypes by this method (Turner and Cherniak, 1991). Also, structural differences have been reported among strains of the same serotype (Cherniak et al. 1991; Cherniak et al. 1993; Small et al. 1986; Turner et al. 1992), and antigenic differences have been identified by monoclonal antibodies (Belay et al. 1993; Dromer et al. 1993; Spiropulu et al. 1989; Todaro-Luck et al. 1989). GXM is poorly immunogenic alone, but if conjugated to a protein carrier induces a strong antibody reponse, as then the whole cell is the immunogen (Casadevall et al. 1992; Devi et al.

1991; Goren and Middlebrook, 1967). GXM has been shown to be anti-inflammatory, antiphagocytic and immunosuppressive. Mouse studies have shown that GXM induces specific tolerance both in a low-dose T-cell- dependant and high-dose T-cell-independant manner (Cherniak and Sundstrom, 1994; Sundstrom and Cherniak, 1993). For some time GXM has been known to be an activator of the alternative complement pathway, resulting in opsonization of encapsulated yeast cells with C3 (Bolanos and Mitchell, 1989). Recently Kozel et al have linked the activation of the alternative pathway with GXM and quantified the deposition of C3b and iC3b (Kozel, 1993; Pfrommer et al. 1993; Young and Kozel, 1993).

In addition to GXM, two further minor carbohydrate antigens, GalXM and mannoprotein form the capsule and can be obtained from culture filtrates of *C. neoformans* (Cherniak et al. 1982; Turner et al. 1984). Mannoprotein is the immunodominant antigen responsible for evoking cell mediated immunity (CMI) (Cherniak and Sundstrom, 1994). GXM and mannoprotein appear to have separate effects on the immune system (Murphy et al. 1988), while at present the role of GalXM is unknown (Cherniak and Sundstrom, 1994).

1.4.3 Influences on capsule size.

Capsule formation is a major feature of *C. neoformans*. Clinical specimens virtually always demonstrate this finding, but on repeated subculture the size of the capsule may be diminished, although optimisation for a given strain can be achieved by manipulating the environmental conditions in which it is grown. The capsular size can be markedly influenced by the environmental conditions. At physiological pCO_2 concentrations capsular size is increased, whereas at environmental concentrations it is diminished (Granger et al. 1985). As the growth rate is slowed so the size of capsule is increased. Culture with 1% glucose, <1.0 μg/ml thiamine, >1.0 mg/ml glutamate, neutral pH, elevated carbon dioxide or decreased iron concentration maximises formation of the capsule (Dykstra et al. 1977; Granger et al. 1985; Littman, 1958; Vartivarian et al. 1993). Reduction of capsule formation can be achieved by cultivation at high osmolarity (16% glucose or 2.9% NaCl) or acid pH, or storage in soil (Dykstra et al. 1977; Ishaq et al. 1968). When grown under identical conditions different strains produce capsules of differing thicknesses, demonstrating the phenotypic variation of *C. neoformans*.

The availability of ferric iron also affects growth and capsule size. With reduced ferric iron it increases (Vartivarian et al. 1993) and it has been suggested that *C. neoformans* has specific iron receptors for iron acquistion (Jacobson and Vartivarian, 1992). Such stimuli may influence adaptation to survival in the host.

1.5 *Cryptococcus neoformans* varieties *gattii* and *neoformans*

The varieties of *C. neoformans* have been defined on the basis of genetic and biochemical studies. *C. neoformans* var. *neoformans* includes serotypes A, D and AD, whereas *C. neoformans* var. *gattii* includes serotypes B and C only. The two varieties can easily be separated on the basis of colour changes on solid media, such as canavinine-glycine-bromothymol blue (CGBB) agar (Plate 1.1), which turns from green/yellow to cobalt blue with var. *gattii*, but not with var. *neoformans*. (Kwon-Chung et al. 1982b).

This method is sensitive, specific and easy, without recourse to sophisticated or expensive equipment, and so is practicable to use in a tropical setting. *C. neoformans* var. *gattii* is resistant to L-canavanine, whereas all serotypes D and 28% of A serotypes are sensitive at concentrations of 5 μ/ml or less (Polacheck and Kwon-Chung, 1986; Kwon-Chung et al. 1982a). The resistant *C. neoformans* var. *neoformans* isolates do not assimilate glycine. A colour change results from the rising pH as *C. neoformans* var. *gattii* assimilates glycine. In addition *C. neoformans* var. *gattii* assimilates maleate and is relatively temperature sensitive (Rippon, 1988; Kwon-Chung et al. 1982a). It seems that the ecology, epidemiology and pattern of disease caused by the two varieties differs.

Plate 1.1 Canavanine glycine bromothymol blue agar used for biotyping. *Cryptococcus neoformans* var. *gattii* turns the agar blue while with var. *neoformans* it remains light green

1.6 Molecular aspects of typing

Molecular methodology is being increasingly applied to cryptococci, and at least 23 *C. neoformans* genes have been cloned, (Mitchell and Perfect, 1995). Pulsed field gel electrophoresis (PFGE) has been used to identify the chromosome size and number (electrophoretic karyotyping) in a number of eukaryotes, and differentiates between the two biotypes of *C. neoformans* (Wickes et al. 1994). The smallest chromosome of var. *gattii* is 400-700 kB in size and that for var. *neoformans* consistently larger about 770 kB (Wickes et al. 1994). Var. *gattii* isolates average 13 chromosomes, and var. *neoformans* average 12. Typing has also been carried out by random amplification of polymorphic DNA (RAPD), by the polymerase chain reaction (Crampin et al. 1993; Haynes et al. 1995) and DNA finger printing of restriction endonuclease digested DNA with dispersed repetitive DNA sequences cloned from *C. neoformans* (Spitzer and Spitzer, 1992; Varma and Kwon-Chung, 1992; Haynes et al. 1995). Restriction fragment length polymorphism analysis in mitochondrial DNA has also been used to differentiate strains and demonstrate varietal-specific patterns of homology (Varma and Kwon-Chung, 1989).

These typing methods seem to be reproducible and discriminatory and hold out promise in helping to define better the ecology and epidemiology of cryptococcosis. It has been suggested that on the basis of biochemical and genetic differences the two varieties of *C. neoformans* may be diverging into separate species. Mating of intervarietal crosses was only successful in so far as serotypes B and D produced 30% viable progeny (Kwon-Chung et al. 1982a).

1.7 Ecology

1.7.1 Natural niche

From the information currently available it seems likely that the two varieties of *C. neoformans* occupy different environmental niches. These are summarised in Table 1.1.

Table 1.1 *Cryptococcus neoformans* isolates from natural sources

Variety	Avian guano	other sources
Cryptococcus neoformans* variety *neoformans*	pigeons	rotting vegetables
	canaries	fruits and juice
	parrots	wood
	budgerigars	dairy products
	swallows	soil
	munia birds	bagpipes
Cryptococcus neoformans* variety *gattii		*Eucalyptus* spp.
		koalas, ?bats

*includes isolates where type not stated.
Adapted from (Kwon-Chung et al. 1990). References: Emmons, 1955; Pal and Mehrotra, 1985; Pal, 1989; Ellis and Pfeiffer, 1990b, Ellis and Pfeiffer, 1990a; Sanfelice, 1894; Cobcroft et al. 1978; Klein, 1901; Swinne et al. 1986a; Lazera et al. 1993

For many years it has been recognised that var. *neoformans* can be found in soil and weathered avian guano, particularly that of pigeons (*Columbia livia*), which is enriched with a high content of low-molecular weight nitrogenous compounds such as creatinine (Emmons, 1955; Emmons, 1951). Staib showed that *C. neoformans* utilises creatinine as a nitrogen source, unlike other species of *Cryptococcus*. Therefore the droppings can serve as a selective medium in nature for *C. neoformans*. Despite the association with avian sources, birds do not become infected, perhaps because of their high body temperature, yet they may distribute the yeasts. In such nesting areas the cells have minimal capsules, are dry (dessicated) and can easily be aerosolized (Bulmer, 1990; Neilson et al. 1977; Ruiz and Bulmer, 1981). These smaller yeast cells could then be inhaled to the alveolus (Neilson et al. 1977; Powell et al. 1972). *C. neoformans* var. *neoformans* has been isolated from droppings of other birds including canaries (Staib, 1962a; Swinne-Desgain, 1975), parrots (Bauwens et al. 1986), munia birds (Pal, 1989), buderigars and other birds (Staib and Heissenhuber, 1989; Wegener and Staib, 1983), swallow's nest, dairy products, soil (Ajello, 1958; Currie et al. 1990), wood, rotting vegetables fruits and in a bagpipe (Cobcroft et al. 1978).

Until relatively recently the natural source of *C. neoformans* var. *gattii* has remained elusive. In 1989 Ellis and Pfeiffer reported the environmental isolation of var. *gattii* in South Australia, New South Wales and subsequently California (Ellis and Pfeiffer, 1990b; Pfeiffer and Ellis, 1991). The organism was isolated from debris (bark, wood and leaves) under the canopies of *Eucalyptus camaldulensis* (red river gum) during and for some months after flowering (Plate 1.2).

Airborne sampling detected fungus around flowering, but not non-flowering trees. Electrophoretic karyotyping of eucalypt and human derived *C. neoformans* var. *gattii* confirm that they are the same organism (Kwon-Chung et al. 1992b). It should be noted that var. *gattii* serotype B but not serotype C has been isolated from environmental sources. *E. camaldulensis* is concentrated in the subtropics and tropics, having been planted widely for commercial purposes. These regions in general correlate with areas of the world where clinically apparent human *C. neoformans* var. *gattii* infections occur (Ellis and Pfeiffer, 1990b). Ellis and Pfeiffer have also reported var. *gattii* carriage in koalas (Ellis and Pfeiffer, 1990a) as well as *E. tereticornis*, (forest red gum), closely related to *E. camaldulensis*, with a similar distribution. This ties in with previously reported cryptococcosis in koalas, which feed on eucalypts (Bollinger and Finckh, 1962). In Brazil, Lazera on one occasion found var. *gattii* in bat guano, and proposed that these mammals may have a role in distribution of this variety in nature (Lazera et al. 1993).

Plate 1.2 ***Eucalyptus camaldulensis*** **in Melbourne Botanical Gardens**

1.7.2 Wood sources

C. neoformans in nature is saprophytic, and its natural niche has been widely sought. It may be that the findings of var. *gattii* in association with Eucalypt sources indicates such a niche. *C. neoformans* has been isolated from wood in an aviary in the Antwerp zoological garden (Bauwens et al. 1986), but examination of 477 samples of tropical wood was negative (Swinne and Ed: Torres-Rodriguez, 1988). It is possible that the telomorphic (sexual) phase is related to a natural niche with wood in the environment. Recently the isolation of var. *neoformans* from decaying wood on the inner surface of hollows in living trees in urban Rio de Janiero, Brazil, has been reported. The trees involved were the Pink shower tree (*Cassia grandis*), November shower (*Senna multijuga*), Fig tree (*Ficus meciocarpa*), Java plum (*Syzgium jambolan*), Munguba (*Bombax munguba*), Coconut palm (*Cocos nucifera*) and mango tree (*Mangifera indica*). Homogenized wood suspension was streaked onto niger seed agar in all instances resulting in positive isolates. All but one of 113 air samplings were negative. Decaying wood is discoloured suggesting partial lignin and xilan degradation. The wood debris from eucalypts contains very high concentrations of lignin and polyphenols suggesting that phenol oxidase activity of *C. neoformans* may be an adaptation to its natural habitat (Ellis et al. 1996).

1.8 Epidemiology - clinical isolates

Kwon-Chung and Bennett have described varieties and serotypes of 725 clinical isolates from patients without the Acquired Immunodeficiency Syndrome (AIDS) (Kwon-Chung and Bennett, 1984). Those isolates from the tropics and subtropics such as Central Africa, Brazil, South East Asia, Australia and Southern California

show a high frequency (35-100%) of *C. neoformans* var. *gattii* (serotypes B and C). In contrast the isolates from temperate climates such as the United States (exluding California and Hawaii), Europe and Japan were virtually all *C. neoformans* var. *neoformans* (serotypes A and D). Of these isolates, 16% were serotype A, 16% D or AD, 11% serotype B and 2% serotype C, and 1% untypeable. Four serotype B isolates came from the United Kingdom, but the details of patient origin were not available, whilst 88% of type C isolates came from Southern California. Dromer et al have found that *C. neoformans* serotype D is relatively more common in France, comprising 21% of clinical isolates. Serotype D seems to be more frequent in the more humid and colder regions amongst patients over 60 years of age, born in Europe, receiving corticosteroid treatment and those who had skin lesions (Dromer et al. 1996).

With the advent of AIDS the geographical distribution of serotypes has not changed, but the relative frequencies of isolates is altering dramatically, particularly in the tropics and subtropics. For example in California, initial reports have suggested that where a large number of clinical isolates were var. *gattii*, all infections in AIDS patients were due to var. *neoformans* (Rinaldi et al. 1986). In Central Africa 92% of isolates collected between 1951 and 1969 were var. *gattii* (Kwon-Chung and Bennett, 1984; Swinne-Desgain, 1976), but only var. *neoformans* was found amongst isolates from Zairean patients between 1970 and 1987 (Kapend'a et al. 1987; Kwon-Chung et al. 1990). Over these years cases of cryptococcosis increased rapidly, with all isoates since 1980 coming from patients with probable or definite AIDS. Var. *gattii* infection in AIDS has been infrequently described, despite the

previous predominance of these infections in the tropical and subtropical areas where now AIDS and var. *neoformans* infections are dominant.

1.9 Epidemiology - patients

In temperate climates cryptococcal meningitis has tended to occur in immunocompromised patients, even before the spread of AIDS. In a UK series of 61 cases, 85% had predisposing disease, and in American studies up to 69% of patients had concurrent conditions associated with immunosuppression, particularly lymphoma, sarcoidosis and corticosteroid treatment (Diamond and Bennett, 1974; Hay et al. 1980; Lewis and Rabinovich, 1972). In such patients the predominant abnormality is an impairment of cell mediated immunity. There has been a suggestion that some apparently immunocompetent patients had minor defects in their T cell response to cryptococcal antigens (Graybill and Alford, 1974; Schimpff and Bennett, 1975; Lalloo et al. 1994). It appears that neither neutropenia nor immunoglobulin deficiency increase susceptibility to cryptococcosis. In contrast to the studies mentioned above, those reported from the Northern Territory of Australia, Zimbabwe, Papua New Guinea (PNG) and Singapore only reported up to 12% of patients with a condition associated with immunosuppression (Lo, 1976; Gould and Gould, 1985; Lalloo et al. 1994; Tay et al. 1972; Tjia et al. 1985). In both tropical and temperate areas men appear to be infected more frequently than women and the incidence of cryptococcosis in prepubescent patients is low. This could be linked to variation in exposure or possible protective effects of oestrogens against cryptococci. Tropical studies do however demonstrate a less marked male

preponderance and younger age group, with a peak incidence in the third decade of life, rather than over the age of 30 as found in temperate series.

With the advent of AIDS the incidence of cryptococcosis has risen dramatically. In the UK 4% of patients with AIDS have developed cryptococcosis while in the United States 6-10% of such patients are infected with *C. neoformans*. (Knight et al. 1993; Chuck and Sande, 1989). In the tropics the number of patients with cryptococcosis may be as high as 30% of all patients with AIDS in some regions (Taelman et al. 1991). This is having massive social and economic repercussions. Virtually all the isolates from these patients, with a few exceptions, have been var. *neoformans*, even where previously var. *gattii* predominated. These variations suggest a difference in the pathogenicity of the two varieties of *C. neoformans*.

In PNG and Victoria, Australia var. *gattii* was not found in immunosuppressed hosts, whereas 87-90% of var. *neoformans* infections occurred in immunosuppressed hosts in the Victorian and another recent Australian study (Naraqi et al. 1979; Lalloo et al. 1994; Speed and Dunt, 1995; Mitchell et al. 1995).

A summary of differences between the two varieties of *C. neoformans*, including epidemiological differences, is given in Table 1.2.

Table 1.2 Probable and possible major differences* between *Cryptococcus neoformans* varieties *gattii* and *neoformans* and resultant central nervous system disease

Feature/Variety	*gattii*	*neoformans*
Smallest chromosome	400-700kB	~770kB
Average number of chromosomes	13	12
Serotypes	B/C	A/D AD
Capsular polysaccharide molar ratios of Xyl:Man:GlcA*	B = 3:3:1; C = 4:3:1	A = 2:3:1; D = 1:3:1
Rate of capsular C3 deposition	slower	faster
glycine assimilation	100%	10-20%
canavanine susceptibility	no	yes
malate assimilation	yes	no
Distribution	Tropics/subtropics	Worldwide
Predominant patient group	Immunocompetent	Immunocompromised.
Features	Cryptococcoma with long term neurological morbidity	Diffuse cerebral involvement without focal deficit
Length of treatment	?longer	?shorter
Optimal outcome	Cure; some are left with residual neurological deficit after treatment	Maintenance therapy - dictated by underlying disease

* These are generalisations; some exceptions exist.
** Xyl = xylose, Man = mannose, GlcA = glucoronic aid
References: (Wickes et al. 1994; Bhattacharjee et al. 1984; Young and Kozel, 1993; Kwon-Chung and Bennett, 1984; Speed and Dunt, 1995; Polacheck and Kwon-Chung, 1986; Sabetta and Andriole, 1985; Mitchell et al. 1995; Speed, 1990; Henderson et al. 1981)

1.10 Virulence

Although it is not known to produce any exotoxins, there are a number of factors that contribute to the virulence of *C. neoformans*. These include the ability to grow at body temperature, 37^0C, as well as at the lower temperatures found in the environment unlike other cryptococcal species, the production of a polysaccharide capsule, superoxide dismutase activity, melanin production and possibly α mating type, as shown in Table 1.3.

Table 1.3 Factors that may play a role in the virulence of *Cryptococcus neoformans*

Virulence Factor	Mechanism Enhancing Virulence
Growth at 37^0C	Ability to survive and grow in deep tissue at body temperature.
Polysaccharide capsule	Antiphagocytic; inhibits lymphoproliferation; T-cell activation and cytokine release; depletes complement levels; potentiates HIV infection.
Superoxide dismutase activity	Confers resistance against microbicidal oxidants.
Melanin production by phenol oxidase	Confers resistance against microbicidal oxidants.
Mating Type (α)	Mechanism undefined.
Mannitol production	Scavenges free radicals, increases heat and osmotic tolerance
Phospholipase production	Uncertain

References: (Bulmer and Sans, 1968; Macher et al. 1978; Truelsen et al. 1992; Mody and Syme, 1993; Wagner et al. 1992; Jacobson et al. 1994; Wang and Casadevall, 1994; Kwon-Chung and Rhodes, 1986; Jacobson and Tinnell, 1993; Kwon-Chung et al. 1992a; Rhodes et al. 1982; Chaturvedi et al. 1996a; Chaturvedi et al. 1996b; Chen et al. 1997)

1.10.1 Capsule in virulence

Encapsulated yeast activates complement solely by the alternative pathway, and once opsonized can be phagocytosed by macrophages, neutrophils and monocytes (Mitchell and Perfect, 1995). Phagocytosis of encapsulated yeasts is reduced in strains with large capsules (Bulmer and Sans, 1968). Differences in the structure of the capsules may influence this activation, impeding clearance, and may contribute to difference in pathogenesis of different serotypes, as C3-binding rates to serotypes B and C are slower than to serotypes A and D (Young and Kozel, 1993). A large load of organisms may cause depletion of complement and opsonin (Macher et al. 1978). The antiphagocytic properties of the capsule may be overcome in vitro by mouse macrophages activated by tumour necrosis factor (TNF) and granulocyte macrophage colony stimulating factor (GM-CSF) (Collins and Bancroft, 1992). Capsular glucoronxylomannan (GXM) has been shown to promote pro and anti inflammatory cytokine release from peripheral mononuclear cells and neutrophils respectively (Vecchiarelli et al. 1996; Retini et al. 1996). Although in vitro interactions therefore occur, the net effect in vivo has yet to be elucidated. Molecular evidence of the importance of the capsule in cryptococcal virulence has been demonstrated by deletion of the CAP59 gene, resulting in acapsulate strains of reduced virulence in mice (Chang and Kwon-Chung, 1994). In this series of experiments restoration of this gene and capsule production resulted in a return of virulence.

1.10.2 Melanin production

The ability to produce melanin-like pigment from catecholamine precursors via a phenoloxidase system is thought to play a role in virulence as mutants deficient in melanin have been shown to be less virulent in mice (Kwon-Chung and Rhodes, 1986). The central nervous system is rich in these precursors, which may account for the predilection of *C. neoformans* for this site, allowing production of melanin and protection from host defences. Melanin can also act as an antioxidant, confering resistance to microbiocidal oxidants such as hypochlorite and permanganate ions. The CNLAC-1 gene, which is responsible for melanin production by encoding laccase enzyme in *C. neoformans* has been isolated and cloned (Williamson, 1994). Mice infected with *C. neoformans* with disrupted CNLAC-1 were not killed, whereas complementation of melanin negative mutants with CNLAC-1 increased virulence to mice (Salas et al. 1996). Recent studies by Huffnagle and colleagues suggest that melanin may act by down regulating the afferent phase of T cell-mediated immunity rather than protecting the organism from intracellular killing (Huffnagle et al. 1995). At 37^0C melanin is supplemented by superoxide dismutase activity, which has a similar though narrower spectrum of activity (Jacobson et al. 1994)

1.10.3 Mating type

The mating type must differ for the sexual process to occur, which prevents mating between genetically identical cells. It is also thought to be associated with virulence. The α-type of *C. neoformans* var. *neoformans* is 30-40 times more common in environmental and clinical isolates than the a-type, and has been shown

to be more virulent in mice (Kwon-Chung et al. 1992a). The molecular basis for these findings has not as yet been clarified.

1.10.4 Mannitol production

C. neoformans isolates produce the acyclic hexitol, D-mannitol in vitro which may be found extracellularly (Wong et al. 1990). It has been suggested that cerebral tonicity and oedema may be caused by accumulated mannitol and that it scavenges free radicals, interfering with cryptococcal killing by phagocytes (Chaturvedi et al. 1996b). Mannitol production also influences *C. neoformans* tolerance to heat and osmotic stresses and its pathogenicity in mice (Chaturvedi et al. 1996a).

1.10.5 Other candidates

In addition to mating type there has been a report of secreted proteinases which may contribute to the breakdown of host tissue or humoral proteins (Brueske, 1986). Further extracellular proteins have recently been found which result in an antibody response (Chen et al. 1997). Extracellular phospholipase activity has now been correlated with virulence in a mouse model resulting in the suggestion that phospholipases may have a role to play in human virulence (Chen et al. 1997).

Other non-capsular antigens have recently been reported by Hamilton et al (Hamilton and Goodley, 1993). The first of these was purified from culture filtrate material and found to be an acidic glycoprotein containing mannose with a size of 115 kDa. This binds a monoclonal antibody that reacts only weakly with the mannoprotein (Hamilton and Goodley, 1993). On immunoblots patient sera reacted

strongly to this antigen. The second is a cytoplasmic antigen which reacts to sera from HIV positive patients and immunocompetent var. *gattii* patients. A 19kDa band was identified as a superoxide dismutase purified and characterized from both varieties of *C. neoformans*. This enzyme is a further candidate as a determinant of virulence in *C. neoformans*.

1.11 Host response

1.11.1 Exposure to *Cryptococcus neoformans*

Where *C. neoformans* is common in the environment, exposure is most likely to be frequent. Surveys using cryptococconin (antigenic extract of *C. neoformans*) for skin testing are difficult to interpret as skin testing has not been standardised, and so comparisons are difficult to make. They do however demonstrate skin reactivity in laboratory personnel who have worked with *C. neoformans* and pigeon handlers (Atkinson, Jr. and Bennett, 1968; Newberry, Jr. et al. 1967). This implies exposure, although there is no evidence of occupationally acquired cryptococcosis other than by direct skin inoculation of blood infected with *C. neoformans* (Glaser and Garden, 1985). Pigeon handlers have a high frequency of cryptococcal antibody in serum. Outbreaks of cryptococcosis have not been described, although there are anecdotal reports of cases asssociated with a particular source (Procknow et al. 1965). While these studies show that exposure to *Cryptococcus neoformans* is common among certain groups, there is no evidence of increased incidence of disease in such groups. Molecular typing techniques have not yet shown exposure to a specific source to be linked with a case of cryptococcosis. However, it is interesting to note that some

clinical and environmental isolates are indistinguishable as determined by restriction fragment length polymorphism analysis (Currie et al. 1994). There is also concordance of clinical and environmental isolates of *C. neoformans* by RAPD analysis (Sorrell et al. 1996).

1.11.2 Pathogenesis

Of crucial importance as to whether any organism invades and causes infection are the virulence of the organism, size of inoculum and the host defences. Integrity of the host defences would seem to be the key to repulsion of *C. neoformans*. This assumption would seem reasonable because of the ubiquity of this yeast in the environment, yet low incidence of symptomatic infection. The implication being that most people, other than those with compromised defences, are probably resistant to such infection. This does not explain the occurrence of disease in those with apparently intact host defences. One suggestion has been that such individuals are exposed to a large inoculum as happens with histoplasmosis and coccidiomycosis (Bulmer and Tacker, 1975). Another explanation may be that different strains and indeed serotypes differ in virulence such that the strain inhaled determines whether infection occurs. Both these concepts may be valid, but there is evidence from experimental and clinical studies that intact cell mediated immunity is of crucial importance in resistance to cryptococcosis.

It is thought that cryptococcosis in humans beings is most likely to be acquired by inhalation of infectious airborne particles. The incubation period is unknown and could be weeks, months or even years; it is uncertain whether latency occurs.

Whether the infectious propagules are desiccated yeast cells (less than 2 microns) (Staib, 1987; Staib and Heissenhuber, 1989) or basidiospores (less than 3 microns) of the sexual state (*F.neoformans*) is unclear (Cohen, 1982; Cohen et al. 1982), but it is thought that the encapsulated yeast cells are too large (4-20 microns) to penetrate the defences of the upper respiratory tract. It seems likely that following inhalation of the infectious propagule, significant clinical illness usually does not occur, but in some cases there is progression to clinical disease.

1.11.3 Cellular immunity

Cellular defences are paramount in containing cryptococci. Mouse studies have shown that neutrophils initially play a significant role in containing pulmonary cryptococcal infection, followed later by monocytes (Gadebusch, 1972). Providing cryptococci are opsonized by complement, phagocytosis by neutrophils may occur, and is more efficient than by monocyte phagocytosis (Miller and Mitchell, 1991). AIDS patients often have chemotactic and phagocytic defects in their neutrophils and monocytes, which could partly explain their greater susceptibility to cryptococcal infection. However, in general patients with neutropenia are not at greatly increased risk. A number of lymphocytic cells are involved in controlling cryptococci. In mice, CD4+ T-cells have been shown to be important in containing central nervous system infection (Hill and Aguirre, 1994). AIDS patients are deficient in $CD4^+$ T cells, and this probably plays an important role in disease caused by *C. neoformans* as shown by the occurrence of cryptococcal infection in patients with idiopathic $CD4^+$ lymphocytopenia (Smith et al. 1993; Duncan et al. 1993). It has been found that the gp120 (the HIV-envelope protein) inhibits alveolar

macrophage ingestion and growth inhibition of cryptococci (Wagner et al. 1992). This may contribute to the infection of AIDS patients by cryptococci. Monocyte interleukin 6 (IL-6) production has been stimulated by *C. neoformans* capsular components (Delfino et al. 1997). IL-6 may play a role in upregulating HIV replication in monocyte cell lines and act synergistically with GM CSF and tumour necrosis factor alpha (TNF-α) in the induction of latent HIV infection (Poli et al. 1990).

In the presence of complement the cytokines TNF-α and GM-CSF have been shown to upregulate cytokine-dependent phagocytosis of encapsulated cryptococci by murine macrophages (Collins and Bancroft, 1992). Inhibiting these cytokines with monoclonal antibodies leads to increased mortality in infected mice. Macrophages incubated with interferon gamma (IFN-γ) and macrophage colony stimulating factor (M-CSF) have increased antifungal activity, which is not due to increased phagocytosis. Monocytes exposed to purified GXM secrete reduced amounts of IL1-β and TNF-α (Vecchiarelli et al. 1995b). However, capsular polysaccharide has been reported to induce proinflammatory cytokine from human PMNs (Retini et al. 1996). Growth inhibition by a nitric oxide (NO)-related mechanism has recently been reported to inhibit cryptococcal growth in human fetal astrocytes, suggesting that NO may be an effector molecule (Lee et al. 1994). Following treatment for cryptococcosis, apparently immunocompetent individuals showed some abnormalitites in delayed skin testing to cryptococconin and other antigens, and leukocyte migration inhibition (Schimpff and Bennett, 1975; Graybill and Alford, 1974; Henderson et al. 1982). There have been early reports of striking

anticryptococcal defects in Natural Killer cells (NK cells) both from HIV-infected patients and apparently non-immunocompromised individuals with cured cryptococcosis (Horn and Washburn, 1995; Washburn, 1996). Unlike the pattern in NK cells from HIV infected patients, the activity of those NK cells from apparently immunocompetent individuals with past cryptococcosis could not be restored by interleukin 12 (IL-12) in vitro. It should be noted that these defects in CMI have been detected following cryptococcal infection, so it is not strictly possible to be certain as to whether they are the result of infection, or predate it. These studies have been carried out in patients infected with *C. neoformans* var. *neoformans*.

1.11.4 Humoral immunity

Antibody and complement are important elements in enhancing cellular host anticryptococcal defences (Mukherjee et al. 1995; Diamond et al. 1972; Levitz et al. 1991; Diamond and Erickson, 1982; Kozel et al. 1984; Miller et al. 1990; Wilson and Kozel, 1992; Levitz and Tabuni, 1991). Patients with cryptococcosis often have low levels of opsonins as activation by the capsule results in depletion of complement (Macher et al. 1978; Truelsen et al. 1992). Despite being used in serotyping, the capsule is also poorly immunogenic (Murphy and Cozad, 1972) but it powerfully activates the alternative complement pathway (Kozel et al. 1991). Acapsular strains also activate the classical pathway, which suggests that the capsule blocks the cell wall components involved in this pathway (Kozel et al. 1991). C3 binds to cryptococci, apparently at a slower rate for *C. neoformans* var. *gattii* than var. *neoformans* (Young and Kozel, 1993). This may be the result of differing capsular structure and contribute to differing pathogenensis. It may be that in

acapsulate strains C3b is the most important opsonic ligand, while in encapsulated strains rapid conversion to iC3b is found, suggesting that it is the more significant ligand (Pfrommer et al. 1993). C5a is able to induce chemotaxis in neutrophils and monocytes and is triggered by both the capsule and cell wall production via the alternative pathway (Diamond and Erickson, 1982). In addition to these soluble anticryptococcal factors it is thought that normal human serum may contain globulins and a high molecular-weight component that inhibits binding to alveolar macrophages (Reiss et al. 1975; Igel and Bolande, 1966; Levitz et al. 1992). There may also be a fungicidal substance present in saliva (Igel and Bolande, 1966).

1.11.5 Central nervous system localization

The apparent predilection of cryptococci for the CNS may be linked to several factors, including the lack of detectable complement activity in CSF, absent or delayed inflammatory response to cryptococci in the brain, CNS presence of catecholamine precursors (dopamine) and D-mannitol production by cryptococci which may contribute to cerebral oedema and interfere with killing by host phagocytes (Wong et al. 1990). Although the inflammatory responses seen elsewhere in the body may not occur in the CNS, endogenous cells such as astrocytes can produce cytokines and nitric oxide and may play a role in controlling cryptococcal invasion (Lee et al. 1994).

1.12 Clinical features

It seems likely that following inhalation, significant clinical illness usually does not occur, but in some cases there is progression to disease, the most serious form of which is meningitis or meningoencephalitis, which is fatal if untreated. The incubation period is unknown and could be weeks, months or even years; it is uncertain whether latency occurs. Virtually any organ may be infected, including bone and soft tissue, generally via haematogenous spread from the lungs.

The clinical features of symptomatic *C. neoformans* infection overlap in different patient groups and patient areas, but significant differences do exist. Many reviews compare findings between those patients with AIDS and without AIDS (Mitchell and Perfect, 1995). These differences tend to reflect the degree and type of immunodeficiency in the bulk of patients without AIDS who are in some way predisposed to significant infection with *C. neoformans*. In general, AIDS patients have a greater burden of yeasts at the sites of infection and relatively few inflammatory cells. There is a high frequency in such patients of positive blood and urine cultures, extraneural sites of infection, many yeast cells yet few inflammatory cells in the CSF and a higher incidence of clinical disease and relapse (Dismukes, 1988; Eng et al. 1986; Kovacs et al. 1985; Zuger et al. 1986). In patients with marked immunosuppression generalized infection occurs including adrenals, heart, liver, lymph nodes, joints and kidneys (Perfect, 1989). The main sites important for diagnosis and management are the lungs, CNS, skin, prostate and eye.

1.12.1 Lungs

It is thought that the normal route of infection is via the lungs, but the form of the infectious propagule is uncertain.

Pulmonary crytpococcosis has been well described; one review indicates well the features that may be found (Campbell, 1966). One third of cases merely have an abnormal chest X-ray. Most patients though present with symptoms. Cough (54%), chest pain (46%), sputum production (32%), weight loss (26%), fever (26%) and haemoptysis (18%) are typical clinical findings (Campbell, 1966). Other rare manifestations are dyspnoea, night sweats and superior vena cava obstruction (Lehmann et al. 1984; Menon and Rajamani, 1976). Presentation may be similar to that of tuberculosis and histoplasmosis, and it is important to investigate appropriately, as co-infection with *Mycobacterium tuberculosis* has been described as has concurrent hydatid disease (Long, Jr. et al. 1980; Riley and Cahan, 1972; Dalgleish, 1981). In immunocompetent hosts the diagnosis of pulmonary cryptococcosis can be made by examination of sputum or lung biopsy material, and antigen detection in conjunction with chest radiography. Often asymptomatic respiratory tract colonization is found in patients with pre-existing lung conditions, such as chronic obstructive airways disease (Hammerman et al. 1973; Subramanian et al. 1982; Tynes et al. 1968). In the immunocompetent host, such colonization does not spread elsewhere and treatment may not be required. It is important to exclude CNS involvement in patients with pulmonary colonization/infection (Kerkering et al. 1981; Lehmann et al. 1984). Pulmonary involvement was found in 57% of cases with meningitis caused by *C. neoformans* var. *gattii* in Northern

Australia (Fisher et al. 1993). Chest X-ray appearances in pulmonary cryptococcosis are variable and the true aetiology of an X-ray abnormality may only become evident when investigations of possible malignancy yield cryptococci (Khoury et al. 1984). Serum cryptococcal antigen, which should routinely be checked in such instances, may not be positive. A positive result could indicate a higher fungal burden and so suggest increased likelihood of dissemination, encouraging antifungal therapy in such cases.

Immunosuppressed pulmonary cryptococcosis

In a review of pulmonary cryptococcosis in the pre AIDS era, 34 of 41 patients had an immunocompromising condition. (Kerkering et al. 1981). In 29 patients the disease disseminated and in all but one of these the patient was immunocompromised. Eighty three percent of immunosuppressed patients had generalised symptoms such as fever (63%), malaise (61%), chest pain (44%), weight loss (37%), dyspnoea (27%), night sweats (24%), cough (17%), haemoptysis (7%) and headache (7%). All patients had abnormalities on pulmonary radiography such as alveolar or interstitial infiltrates, coin lesions, masses, cavitary lesions and pleural effusions. Meningeal spread occurred in 25 patients within 20 weeks of diagnosis, supporting the recommendation that specific treatment should be instituted in immunocompromised patients. In AIDS, virtually every patient has symptoms such as fever (81%), cough (63%), dyspnoea (50%), loss of weight (47%), headache (41%) and sometimes pleuritic chest pain and haemoptysis (Cameron et al. 1991; Clark et al. 1990b). Some patients may present with the adult respiratory distress syndrome (Stern et al. 1988; Perla et al. 1985; Similowski et al. 1989). Chest X-rays

rarely show nodular and alveolar infiltrates, large masses or pleural effusions, but most frequently reveal focal or diffuse infiltrates and lymphadenopathy (Cameron et al. 1991; Clark et al. 1990b; Miller, Jr. et al. 1990). These appearances are also consistent with those of *Pneumocystis carinii* pneumonia, the most common cause of such findings in AIDS, requiring appropriate investigation (Loerinc et al. 1988). Concurrent oral candidiasis is also a frequent finding highlighting the association with a CD4 count of <200 cells per μl (Crowe et al. 1991). Dissemination to the CNS or blood occurs in about 94% of AIDS patients and so thorough evaluation of a patient with pulmonary cryptococcosis is warranted (Cameron et al. 1991).

1.12.2 Meningoencephalitis

The onset and course of CNS disease are those of a subacute meningitis or meningoencephalitis with headache, fever, lethargy, coma, personality changes and memory loss over a few weeks. There is considerable variation in how patients present; for instance there may be severe headaches of a few days duration, intermittent headaches over months or no headaches. Headache may lead to an earlier diagnosis and so better prognosis (Dismukes et al. 1987).

In tropical series of cryptococcosis, cryptococcal meningitis is the best described form of this infection, probably because it is the most severe manifestation and relatively clear-cut to diagnose. The pattern of disease in tropical and sub-tropical regions and temperate regions varies, probably reflecting the differences in biotype and immunocompetence in the series reported. In Europe and much of the US, even before AIDS, disease tended to occur in immunocompromised patients. In tropical

series the mean time to presentation from onset of symptoms varies from 4-10 weeks, whereas in 'Western' societies presentation is more rapid and disseminates more often, probably related to the higher proportion of immunocompromised patients (Lalloo et al. 1994; Hay et al. 1980; Lo, 1976; Gould and Gould, 1985; Kerkering et al. 1981). In Zimbabwe the mean time from onset to presentation of symptoms was 10 weeks and most Australian patients presented after over a month of symptoms (Lo, 1976; Gould and Gould, 1985) In Western societies disease presents more rapidly and disseminates more often (Hay et al. 1980; Kerkering et al. 1981). Despite these differences, most clinical features are similar in both temperate and tropical regions. Common symptoms and signs of meningitis including headache, nausea, vomiting, fever, photophobia and meningism are accompanied by anorexia, weight loss, visual disturbances and cranial nerve abnormalities, particularly papilloedema or papillitis, which occur in up to 88% of patients (Richardson et al. 1976). The clinical features and outcome of var. *gattii* infections may differ from those caused by var. *neoformans* (Speed and Dunt, 1995; Mitchell et al. 1995; Speed, 1990; Henderson et al. 1981). In two studies patients of similar immunological status were compared (Mitchell et al. 1995; Henderson et al. 1981). In a retrospective comparison of immunocompetent hosts Mitchell et al found that a higher incidence of cryptococcal meningitis in males, and cerebral mass lesions, hydrocephalus, pulmonary-mass lesions and worse outcome were associated with var. *gattii* infection (Speed, 1990). This series reported that a good outcome was statistically more likely in immunocompetent patients with *C. neoformans* var. *neoformans* than var. *gattii* infections, although two of the deaths in the latter group were not directly related to cryptococcosis. In a retrospective series of 133 patients

from the state of Victoria in Australia, there were 71 biotyped isolates (Speed and Dunt, 1995). There were 5 immunocompetent patients infected with *C. neoformans* var. *neoformans*, and all 20 patients infected with *C. neoformans* var. *gattii* were immunocompetent. None of the 20 immunocompetent patients with var. *gattii* died and there was a higher incidence of CNS cryptococcoma (cryptococcal mass focus of infection) and non-fatal complications than in the patients with var. *neoformans* infections (Speed and Dunt, 1995). An update on this second study suggests that while the host preferences of var. *gattii* and var. *neoformans* contrast, the clinical findings in hosts of similar immunological status is not so distinct (Speed, 1996).

1.12.3 Ocular involvement

C. neoformans infection with eye involvement is not infrequent. Prior to the HIV epidemic 45% of all patients with meningitis had ocular signs and symptoms (Okun and Butler, 1964). Ocular palsies devlop as well as retinal involvement, sometimes with concurrent infection with HIV and Cytomegalovirus (Doft and Curtin, 1982). Catastrophic visual loss in patients without endophthalmitis is now well recognised in patients with either normal retinas or papilloedema on fundoscopy (Johnston et al. 1992; Rex et al. 1993). Two major pathogenic processes have been implicated. These are direct involvement of the optic nerve infiltrated by cryptococci and the effects of raised intracranial pressure, however optic nerve arachnoiditis leading to nerve infarction may also occur (Okun and Butler, 1964; Cheong et al. 1988; Rex et al. 1993; Lipson et al. 1989; Kupfer and McCrane, 1974). The distinction betweeen acute and slow visual loss has been made (Rex et al. 1993). Acute visual loss has been attributed to direct optic nerve involvement, and the slow visual loss to the effects of raised intracranial pressure. If the latter is the case, then early intervention to lower intracranial pressure may reduce the outcome in some patients. Lowering of intracranial pressure can be achieved by manoeuvres such as performing sequential lumbar punctures and ventriculoperitoneal shunting, which have been reported to improve visual function, and acetazolamide which has been used in two patients with AIDS and cryptococcal meningitis (Cheong et al. 1988; Denning et al. 1991b; Johnston et al. 1992)

1.12.4 Other sites

Sites beyond the CNS are frequently also involved in cryptococcosis. The protean skin manifestations include acneform lesions, purpura, papules, superficial granulomas, and more recent reports in high risk immunosuppressed patients of molluscum contagiosum-like lesions and cellulitis. (Sarosi et al. 1971; Schupbach et al. 1976; Pema et al. 1994) It is important to biopsy material from new skin lesions in high risk patients, especially as the lesions may herald the onset of systemic disease. Direct inoculation in clinical and laboratory accidents have occurred, producing a papule with or without a local immune reaction (Glaser and Garden, 1985). In males the prostate gland is well recognised as a focus of infection (Braman, 1981), but its greatest significance may be in AIDS patients who frequently have evidence of viable cryptococci in seminal fluid and urine at the end of therapy (Larsen et al. 1989; Staib et al. 1989b). It may be that this site acts as a reservoir of infection and contributes to relapse in some patients. In non AIDS patients this source has been less well studied.

1.13 Diagnosis

1.13.1 Introduction

The diagnosis of cryptococcosis is made by isolation of *C. neoformans* in conjunction with the clinical setting and detection of serum and cerebrospinal fluid capsular antigen by latex agglutination or enzyme immunoassay (Bennett and Bailey, 1971; Horgan et al. 1990; Frank et al. 1993). Routine laboratory tests such

as haematocrit, peripheral white blood cell count and erythrocyte sedimentation rate are often normal.

1.13.2 Antigen and Antibody detection

Latex agglutination detection of cryptococcal capsular polysaccharide was first described in 1963 by Bloomfield and colleagues (Bloomfield et al. 1963). Interference in sera containing rheumatoid factors leading to false positives was abolished by treatment of sera with dithiothreitol or a protease (Stockman and Roberts, 1983). Rarely false positives may occur in the presence of *Stomatococcus mucilaginosis* or *Trichosporon beigelii* (Chanock et al. 1993; McManus and Jones, 1985). Antigen detection is positive in at least 90% of cases with cryptococcal meningoencephalitis, although infection at other sites less frequently results in a positive antigen test (Powderly et al. 1992; Chuck and Sande, 1989; Saag et al. 1992; Bennett and Bailey, 1971). In one study 9% of AIDS patients had culture positive cryptococcal meningitis but negative CSF antigen detection tests although serum was negative in only 1% of patients (Chuck and Sande, 1989). Low levels of antigen, presence of immune complexes (in non-protease treated serum samples), high titres (due to a prozone effect - high concentrations of bound antibody antigen complex inhibit antigen detection, but antigen can be detected at higher dilutions) or poorly/non-encapsulated strains may contribute to such negative antigen tests (Currie et al. 1993; Sadamoto et al. 1993). There are a number of commercial kits available to detect cryptococcal capsular antigen by latex agglutination and enzyme immunoassay, although there is some variation in performance (Tanner et al. 1994). In one study, 4 kits were compared with CSF culture in patients with cryptococcal

meningitis. The sensitivity in CSF ranged from 93-100% and specificity from 93-98% while in serum sensitivity ranges were 83-97% and specificity 95-100% (Tanner et al. 1994). It should be noted that meningitis cannot be excluded simply on the basis of negative (even repeatedly so) CSF and serum antigen tests or India ink stain.

Even when testing is available, antibody levels are not usually raised in patients with cryptococcosis and can be detected in some healthy people, probably as a result of previous exposure (Mitchell and Perfect, 1995). It is possible to detect antibody in convalescent patients.

1.13.3 Microscopy

Rapid and presumptive identification can be made by using the India ink stain on body fluids which highlights the cryptococcal capsule. This should be preceded by gram stain to exclude bacterial pathogens and confirm yeast morphology. The yeast cells are typically spherical and 5-7 μm in diameter. They may have a bud, attached by a thin connection to the body of the yeast. If available, aspirates and similar specimens can be effectively treated with a solution of calcofluor white and examined under a fluorescent microscope (Hageage and Harrington, 1984). In tissue sections methenamine silver and periodic acid-schiff help identify the yeast, although it may appear collapsed and distorted. The capsule can be identified by staining with Mayer's mucicarmine (Mitchell and Perfect, 1995) which colours it rose red while as a further adjunct the Masson Fontana silver stain can be used for melanin, confirming a diagnosis of cryptococcosis (Kwon-Chung et al. 1981).

1.13.4 Isolation

Acapsular mutants will not be identified using this stain, nor by methods which depend on detection of capsular antigen, so extended culture at 30^0C is crucial in establishing the diagnosis in this instance. Isolation is possible on most routine bacteriological or mycological media such as blood, Sabouraud's dextrose and malt extract agars. For nonsterile specimens media containing cycloheximide should not be used because the organism is susceptible to this agent. Niger seed (bird seed) agar is useful for primary isolation from sputum and urine specimens (Denning et al. 1990). Biochemical testing can be used to confirm the identity of any yeast grown.

1.13.5 Cerebrospinal fluid findings

The CSF opening pressure is often raised, as is the protein concentration, accompanied by a depressed glucose level and an abnormal leukocyte count with a lymphocyte predominance. The CSF may sometimes be acellular in immunosuppressed patients, such as those with AIDS (Powderly et al. 1992; Chuck and Sande, 1989; Clark et al. 1990a). Skin testing does not have a role in diagnosis.

1.13.6 Recovery from distant sites

Culture of *C. neoformans* from any site such as skin, bone marrow and urine requires a search for infection elsewhere. It is sometimes necessary to culture sediments repeatedly to obtain a positive culture, as there may be a few cryptococci in the CSF itself. Blood culture is best done by lysis centrifugation (Tarrand et al. 1991), if available, but other techniques do detect yeasts, and can be optimized by venting, extended incubation and terminal subculture. In the future genetic probes may have a role in enabling rapid direct detection of fungal infection (Meyer et al. 1993; Polacheck et al. 1992).

1.13.7 Radiology

Usually chest -Xray is performed as part of the clinical workup in patients with CNS symptoms and may identify another focus of possible cryptococcal infection as well as other pathologies. If there is a reduced level of consciousness or focal neurological findings computed tomography (CT) or magnetic resonance imaging (MRI) is indicated. These are suitable for defining the ventricular system and identifying hydrocephalus, which may need active management.

1.14 Differential Diagnosis

Other conditions that present with symptoms and signs of subacute meningitis may mimic CNS cryptococcosis. These include both infectious and non-infectious causes such as tuberculosis, other deep mycoses, syphilis, brucellosis, sarcoidosis and neoplasm (primary or metastatic). Investigations such as treponemal serology and neuroradiological imaging may help clarify the diagnosis in some instances, but

often positive exclusion and diagnosis by appropriate sampling and culture is the only way to be reasonably sure of the aetiology, and to exclude other pathologies.

1.15 Treatment

1.15.1 Early days

Prior to the discovery of amphotericin B and its commercial availability, cryptococcal meningitis was always fatal. Now after over 35 years experience with amphotericin B it is a curable infection (Sarosi et al. 1969). Previously, various remedies were tried without notable success. These included iodides, penicillin, sulphanilamides, X-ray therapy, hyperthermia and acriflavine but all were unsuccessful. These are reviewed by Cox and Tolhurst (Cox and Tolhurst, 1946) who included an account of immune rabbit serum delivered intraspinally (Shapiro and Beal, 1925). This treatment was discontinued after a possible allergic reaction to the serum. In 1946 Cox and Tolhurst described 12 cases of torula meningitis from southern Australia. Four of these were treated with parenteral autogenous torula vaccine and in two patients it is possible that a temporary remission was induced. Recently this mode of therapy has been revisited by an account of two patients treated with serum therapy in 1963 (Gordon and Casadevall, 1995). This account has highlighted the possibilities of immunotherapy and we now are at a point where vaccine and cytokine therapy are becoming feasible. A recent trial using GM-CSF as adjunctive therapy to amphotericin B in AIDS has suggested that CSF sterilisation is accelerated in the presence of GM-CSF (Torres, C. et al. 1993).

As knowledge and technology advance these cytokines may become real options in therapy.

Before the advent of AIDS, treatment of cryptococcal meningitis with amphotericin B alone was successful in 60-70% of cases (Bennett et al. 1979; Dismukes et al. 1987; Sarosi et al. 1969; Utz et al. 1975). Once flucytosine became available and was given concurrently, success rates rose even further (Bennett et al. 1979). In recent years the triazoles fluconazole and itraconazole have become available and been successfully used in therapy for cryptococcal meningitis. Their relatively low host toxicity, oral bioavailability and attractive pharmakocinetics have lead to popularity with clinicans and their patients, although amphotericin B with flucytosine remains the gold standard treatment. The optimal conditions for triazole administration remain to be defined. Lipid associated formulations of amphotericin B may have a place given their low toxicity. Finally there are intriguing reports of the clinical use of garlic treatment and recently echinocandins and pneumocandins in fungal disease, but their role remains to be defined for cryptococcal meningitis. Possible regimens are discussed in 1.15.5 below.

1.15.2 The antifungal agents

1.15.2.1 Amphotericin B

Derived from *Streptomyces nodosus*, this antifungal is lipophilic and rodlike, acting by insertion into the fungal cytoplasmic membrane (Balakrishnan and Easwaran, 1993). It binds to sterols in the membrane such as ergosterol, increasing membrane permeability resulting in loss of intracellular potassium (Hsu and Burnett, 1993). This and other molecules are lost and fungal viability is impaired. Amphotericin B deoxycholate is administered to patients in 5% dextrose over 2-4 hours. In serum the deoxycholate separates from the amphotericin B, 95% of which binds to serum proteins, presumably bound to cholesterol in such proteins. Degradation occurs in situ, with a small percentage of the drug being excreted in bile and urine. (Block et al. 1974; Craven et al. 1979). Levels are not influenced by hepatic or renal failure. It has been found that with either normal or inflammed meninges penetration is poor.

The glomerular filtration rate is decreased by amphotericin B in a dose dependant fashion caused by a vasoconstrictive effect on afferent renal arterioles, which reduces glomerular and renal blood flow (Sawaya et al. 1991) Other renal effects are potassium and bicarbonate wasting and reduction in erythropoetin synthesis. Permanent destruction of renal tubular cells and loss of nephron units is related to the total dose of amphotericin B. Some clinicians give a litre of normal saline daily which may reduce nephrotoxicity in some patients (Branch, 1988). Due to the

marked potassium losses oral or intravenous replacement is required to prevent hypokalaemia.

As amphotericin B is infused patients may experience chills, fever and tachypnoea which wane over 2-4 hours. Such reactions do not constitute anaphylaxis and are not contraindications for further administration, as they become milder with further infusions. The reactions are less frequent in children and patients on adrenal corticosteroids and may be reduced by premedication with oral paracetemol or 25-50mg hydrocortisone (Bennett, 1995).

There are three lipid associated formulations of amphotericin B licensed in the United Kingdom. Amphotericin B colloidal dispersion (Amphocil) contains cholesterol sulfate, amphotericin B lipid complex (ABLC, Abelcet) is a complex with lipid and liposomal amphotericin B (Ambisome) is a unilamellar liposome containing lipid. These have all been used in the treatment of cryptococcal meningitis (Begue and Lindo-Soriano, 1991; Hostetler et al. 1992; Leenders et al. 1997). In addition to these formulations some hospitals have mixed amphotericin B with Intralipid (Pharmcia and Upjohn), a parenteral fat emulsion (Joly et al. 1996). These new formulations have rarely been compared directly with amphotericin B. One recent randomised trial in patients with AIDS and cryptococcal meningitis suggested that initial treatment with Ambisome 4mg /d was as effective and less toxic than with amphotericin B 0.7mg/d (Leenders et al. 1997). Lipid associated preparations are generally accepted to be less toxic, allowing tolerance of higher

doses, however their efficacy over amphotericin B has not been proven. There is considerable uncertainty over their place and little doubt of their higher cost.

1.15.2.2 Flucytosine

Flucytosine is also known as 5-fluorocytosine (5-FC) and acts as a pyrimidine antagonist. It was originally synthesized as an antitumour agent, but was unsuccessful in this role. Screening revealed that it had antifungal effects which is now its only clinical indication. The fluorine analogue of cytosine, a normal body constituent, it is thought to be deaminated to 5-fluorouracil (5-FU) and then converted to a noncompetitive inhibitor of thymidylate synthetase, which interferes with DNA synthesis (Diasio et al. 1978). It is well absorbed orally, with 90% being exceted in the urine with minimal protein binding (Block et al. 1974). CSF concentrations are about 74% of those in the serum, while peritoneal and haemodialysis remove flucytosine from the body. Hepatic dysfunction does not influence half life, but in renal impairment this may be considerably prolonged.

Toxicity is relatively rare, and includes rashes, diarrhoea and in up to 5% hepatic dysfunction in patients with otherwise previously normal gastrointestinal, renal and haematological function. In patients who have renal failure or are receiving concurrent amphotericin B, marrow suppression and enterocolitis may occur, especially if flucytosine levels in blood exceed 100-125 μ/ml (Stamm et al. 1987). Those experiencing marrow or gastrointestinal toxicity often tolerate lower doses. In rats flucytosine is teratogenic and should not normally be used in pregnancy in humans.

The use of flucytosine alone usually leads to secondary drug resistance with associated clinical failure, so use in combination with amphotericin B is the norm. These two agents are at least additive in vitro and in mice infected with *Candida* and *Cryptococcus* sp. (Medoff et al. 1971; Polak, 1978). Using them together prevents the emergence of flucytosine resistance and permits the effective use of lower and less toxic doses of amphotericin B These benefits and the combined efficacy have been confirmed in large multicentre trials of cryptococcal meningitis treatment (Dismukes et al. 1987).

1.15.2.3 Triazoles

A number of organic compounds have acquired antifungal activity with the addition of an imidazole ring. N-substitution of such imidazoles generates the family of triazoles, which have less effect on human sterol metabolism. They inhibit the 14-α -methylation of lanosterol in fungi by binding to a cytochrome P-450 enzyme. This results in reduced production of ergosterol, essential for normal fungal cytoplasmic membrane. P-450 enzyme inhibition can lead to disruption of cortisol and testosterone in mammals and so selection of antifungals which affect fungal sterol synthesis at much lower concentrations than mammals is an important aspect of triazole drug development. Miconazole was used for treatment of cryptococcosis but its role has been taken over by the newer triazoles. Ketoconzole was initially used for deep fungal infection. Standard doses alone have not been effective in treating cryptococcal meningitis although it may have a role in non meningeal

cryptococcosis (Dismukes et al. 1983). It has largely been superceded by fluconazole and itraconazole.

1.15.2.3.1 Fluconazole

This triazole is well absorbed form the gastrointesinal tract, with over 80% bioavailability (Zervos and Meunier, 1993). Some 60-75% of the oral dose is excreted in the urine and 8-10% in the faeces. Eleven percent of the serum fluconazole is protein bound with the CSF concentration being 70% of that found in serum, whether or not the meninges are inflamed. Penetration into other body fluids is also excellent (Zervos and Meunier, 1993). The levels of phenytoin, glypizide, tolbutamide and cyclosporin are increased by fluconazole, while rifampicin lowers the fluconazole blood levels by a quarter (Weinroth and Tuazon, 1993b). Rarely are there side-effects. These occur in less than 5% of cases and may include nausea, abdominal pain, diarrhoea, vomiting and rash (Perfect et al. 1992). There is uncertainty whether hepatic necrosis may have occurred in patients on fluconazole and alopecia has been reported in those on doses of 400mg daily or higher (Weinroth and Tuazon, 1993). There is no evidence of teratogenicity in rabbits. Oral and intravenous preparations are available for use in fungal infections.

1.15.2.3.2 Itraconazole

At present only oral formulations are available, a suspension has recently been licensed in the United Kingdom with a higher bioavailability than the capsule. Oral absorption is increased in the presence of food (Barone et al. 1993) giving a bioavailability of 55% following breakfast. In the presence of a raised gastric pH as with antacids, H_2 blockers and proton pump inhibitors absorption is substantially impaired (Lim et al. 1993). It is 99% bound to serum proteins, metabolized in the liver and excreted in faeces as metabolites, with no active drug present in the urine. Plasma concentrations are unaffected in renal impairment and dialysis. In tissue itraconazole concentrations are generally higher than in plasma, but in CSF they are generally unmeasureable.

Dose related nausea and abdominal discomfort rarely are severe enough to require stopping therapy and may be reduced by dividing the daily dose. At doses of 400mg daily hypokalaemia and oedema may become evident and an allergic rash is seen occasionally. There does not appear to be hepatotoxicity, but itraconazole is contraindicated during pregnancy and lactation.

Aside from the gastric interactions mentioned above, itraconazole has a number of other clincially significant drug interactions. Levels of itraconazole may be decreased by rifampicin, phenytoin and carbamazepine, while rifampicin levels are themselves reduced by itraconazole. The antifungal can increase levels of cyclosporin, digoxin, terfenadine, astemizole and loratidine. The latter three are

new non sedative antihistamines; accumulation of these may result in life-threatening torsades de pointes (Crane and Shih, 1993).

Itraconazole has a role in treatment of blastomycosis and histoplasmosis (Dismukes et al. 1992), and has been given for indolent cases of invasive aspergillosis (Jennings and Hardin, 1993) and cryptococcal meningitis in AIDS patients (Cleary et al. 1992), but controlled comparisons with amphotericin B have not been carried out.

1.15.2.4 Others

1.15.2.4.1 Garlic

There are clinical reports of garlic (*Allium sativum*) use in the treatment of cryptococcal meningitis, both alone (Anonymous, 1980) and in conjunction with amphotericin B (Tjia et al. 1985; Yu et al. 1988; Davis et al. 1990). There are though no trials reported of garlic treatment in humans. Low concentrations of *Allium sativum* extract are inhibitory and lethal to numerous strains of (untyped) *C. neoformans* (Fromtling and Bulmer, 1978). In vitro combination of amphotericin B and diallyl trisulphide (a Chinese commercial garlic preparation) demonstrated synergistic activity against *C. neoformans* (Shen et al. 1996). Used alone in mice oral garlic reduced brain cryptococcal burden, but did not achieve complete eradication (Louria et al. 1989). In humans commercial *Allium sativum* extract given intravenously to 5 patients with cryptococcal meningitis lead to a doubling of plasma anti *C. neoformans* activity and detectable activity in 4 of 5 CSF samples (Davis et al. 1990). There was no activity in pooled normal CSF.

1.15.2.4.2 Echinocandins and pneumocandins

Echinocandins and pneumocandins, new classes that target the fungal cell wall, are in clinical development. Recently in an in vitro study of activity against fungi, echinocandins were found to be inactive against *C. neoformans* (Zhanel et al. 1997).

1.15.2.4.3 Cytokine therapy

In paediatric AIDS human recombinant GM-CSF has been used inconjunction with liposomal amphotericin B to good effect and neutrophils from patients with AIDS exhibit increased in vitro *C. neoformans* killing (Manfredi et al. 1997a; Vecchiarelli et al. 1995a). Therefore in some circumstances cytokine therapy may be a useful adjunct to treatment.

1.15.3 Susceptibility testing

Initial isolates of *C. neoformans* generally have low minimum inhibitory concentrations (MICs) to amphotericin B, flucytosine and the azoles (Ghannoum et al. 1992). Various methods of sensitivity testing have been proposed, with problems in reproducibility of results even between laboratories using identical methods. In the United States a method using broth macrodilution for yeast testing using standardized media, inocula, and endpoint determinations has been proposed (Espinel-Ingroff et al. 1992; National Committee for Clinical Laboratory Standards. 1992). In RPMI, some strains of *C. neoformans* do not grow well and require 48-72 hours incubation before final MIC determination can be made. Microtitre methods have also been developed using BYNB-7 medium (Ghannoum et al. 1992). It is

hoped that as experience grows with MIC testing in vitro for *C. neoformans*, it will become useful in guiding clinical decisions.

Less than 2% of *C. neoformans* strains are resistant to flucytosine and a rising MIC in isolates correlated with clinical relapse in early studies where flucytosine was used alone for treatment of cryptococcal meningitis and pulmonary cryptococcosis (Utz et al. 1972; Kerkering et al. 1981).

The MICs of amphotericin B to clinical isolates are low and show little variation. Rare cases have been resistant in vitro (Bodenhoff, 1968). It appears that with current treatment regimens failure has not been linked to polyene resistance even in heavily immunosuppressed patients.

The use of azoles in treatment of fungal infections has accelerated with the AIDS epidemic. In vitro activity against primary isolates is usual. With widespread use of azoles in such patients, isolation of resistant *Candida* species have predicted clinical failure. Clinical relapses of patients on cryptococcal therapy have not generally been linked to more resistant isolates (Casadevall et al. 1993). In this study sequential isolates from patients with relapsed cryptococcal meningitis were no more resistant than the initial isolates. Molecular typing of the sequential isolates has indicated that these were relapses rather than reinfections (Spitzer et al. 1993). Often the waning immunity of the AIDS cases has been the most important factor in relapse, although in some instances difficulties in compliance may play a role.

Recent case reports correlating clinical relapse with a rising MIC of fluconazole in AIDS associated cryptococcal meningitis describe rises in MIC from 4 to >64mg/ml, 16 to 128mg/ml and <0.25 to 16 mg/ml (Paugam et al. 1994; Birley et al. 1995). It has been suggested that the reason why this clinical failure linked to resistant isolates occurs infrequently is because the azole resistant strains may be less virulent than the wild-type strains (Iwata et al. 1990).

It would appear that virtually all primary isolates of *C. neoformans* are susceptible to the antifungals used in clinical practice. However it is probably worth storing such isolates so that they can be compared with any future isolate in case of relapse (Mitchell and Perfect, 1995). In such cases an increase of MIC >4 fold would suggest that relapse is related to drug resistance and so a new drug regimen indicated (Mitchell and Perfect, 1995).

Combination testing of antifungal drugs in vitro is even less well standardized for *C. neoformans* than for single agents. Overall it is suggested from in vitro and in vivo studies that a beneficial interaction occurs with flucytosine and azole, amphotericin B with flucytosine, azole, rifampicin, and triple combination of amphotericin-flucytosine -azole. (Borgers and Van de Ven, 1987; Polak et al. 1982). Antagonism has not often been found in vitro in such combinations.

1.15.4 Animal Models

Most of the above observations on sensitivity testing have been made in vitro, but animal models form an important bridge between drug screening and use in human trials. The mouse model has been widely used as mice are genetically stable, their immunology has been well described and most strains are extremely susceptible to disemminated infection as are immunocompromised patients. Infection has been introduced by intrarespiratory, intravenous and intracerebral routes allowing antifungal agents to be tested and compared. Immune based treatments can also be tested in such models, such as TNF-α and GM-CSF (Collins and Bancroft, 1992). Rabbits have been used as a model of cryptococcal meningitis which closely parallels infection in humans (Perfect et al. 1980). In the sphere of antifungal treatment low levels of CSF amphotericin B and itraconazole have been found in both rabbits and human cryptococcal meningitis, yet clinical success has followed treatment using both agents (Perfect and Durack, 1985; Denning et al. 1989). These findings suggest that the action of these drugs is similar in both rabbits and humans and gives confidence in the rabbit as an appropriate model.

1.15.5 Antimicrobial treatment of cryptococcal meningitis in humans

The aim of treatment is to treat symptoms and signs and suppress or eradicate infection. In the immunosuppressed patient the outcome depends on the underlying immunosuppression. If immunosuppression cannot be reduced patients must remain on lifetime prophylactic therapy, while in the immunocompetent the aim is to eradicate infection and allow survival off therapy. Based on studies carried out in areas where *C. neoformans* var. *gattii* is rare, the initial treatment of choice in

immunocompetent patients has been a 6 week course of amphotericin B (0.3mg/kg/day) and flucytosine (150mg/kg/day) (Bennett et al. 1979). The study on which this regime is based was carried out in the pre AIDS era and with the advent of AIDS, therapy has evolved further.

In AIDS patients in the United States a randomised trial of higher dose amphotericin B (0.7 mg/kg/d with or without flucytosine (100 mg/kg/d) over 2 weeks followed by fluconazole (400 mg/d) or itraconazole (400 mg/d) concluded that amphotericin B and flucytosine is associated with an increased rate of cerebrospinal fluid sterilization compared with earlier studies (van der Horst et al. 1997). A subsequent trial to evaluate the role of these two azoles in maintenance therapy using 200 mg/d after successful acute treatment was stopped due to significantly more relapses in the itraconazole arm. Analysis of the data showed that lack of flucytosine during the initial two weeks of therapy was more predictive of relapse than any other factor, including itraconazole maintenance therapy. Many investigators suspected that initial better clearance of the organism is achieved when flucytosine is used during the first two weeks (van der Horst et al. 1997). Currently amphotericin B in combination with flucytosine for 2 weeks, followed by fluconazole 400 mg/d for 8 weeks (reduced to 200 mg/d thereafter) is considered the treatment of choice. Itraconazole is an acceptable alternative treatment for those intolerant of fluconazole, followed by maintenance dose of 400 mg/d. Attention must also be given to reducing raised intracranial pressure, which is associated with a poor outcome (van der Horst et al. 1997). In AIDS patients without poor prognostic indicators, fluconazole 200-400 mg/day has been shown to be as effective as

amphotericin B 0.4-0.6 mg/kg/day (Saag et al. 1992). Fluconazole combined with flucytosine for oral treatment of cryptococcal meningitis in AIDS has given promising results and triple therapy with these two agents and amphotericin B is also reported to have a good outcome when compared with amphotericin B and flucytosine (Larsen et al. 1994; Just-Nubling, 1996).

In patients with features associated with a good prognosis at presentation (headache, normal mental status, CSF leukocyte count of $>20/mm^3$, pretreatment serum cryptococcal antigen titres of <1:32 and post treatment serum and CSF cryptococcal antigen titres of <1:8) up to 88% of patients without immunosuppression will respond to four weeks of therapy (Dismukes et al. 1987). Where possible, flucytosine levels should be monitored.

In immunocompetent and var. *gattii* cryptococcosis there are no trials comparing treatments. In vitro studies comparing susceptibilities of the two varieties to different antifungals have shown largely similar sensitivities, although one showed diminished var. *gattii* sensitivity to flucytosine (Ellis and Pfeiffer, 1992; Shadomy et al. 1987; Fromtling et al. 1986). It has been suggested that in immunocompetent patients with *C. neoformans* var. *gattii* infection, cerebral cryptococcomas or dilated ventricles, a longer course of amphotericin B with flucytosine therapy was required than in immunosuppressed patients (Mitchell et al. 1995). The role of azoles especially in immunocompetent patients is uncertain.

Liposome associated amphotericin B preparations with reduced toxicity have been reported to be efficacious in doses of 3-5 mg kg/day and may have a role where nephrotoxicity is problematical (Schurmann et al. 1991). If host immunosupppression cannot be significantly reduced, life-long maintenance therapy is required to prevent relapse, as in those patients with AIDS.

In practice, treatment and maintenance must be carefully tailored to each individual, depending on severity of disease and underlying host status, with aggressive management of raised intracranial pressure (van der Horst et al. 1997).

Reports of garlic use in the tropics and China are intriguing. There is a report of oral and intravenous garlic preparations alone curing 6 of 21 patients of central nervous system cryptococcosis in China, and of concurrent use of garlic tablets with amphotericin B and flucytosine in 13 patients in Singapore (Anonymous, 1980; Tjia et al. 1985). In another study of two patients with cryptococcal meningitis anticryptococcal activity rose following an infusion with commercial allium sativum (garlic) extract (Davis et al. 1990). The biotypes of the Chinese strains are unknown, whilst in tropical centres where garlic has been used it is likely that var. *gattii* predominates. Perhaps this biotype is particularly sensitive to garlic, or garlic enhances immunity explaining its apparent therapeutic success. Garlic is both cheap and readily available even in less wealthy countries so exploration of its role in treatment of cryptococcosis an attractive avenue of research.

Cytokine therapy may also prove to have a role - there is a preliminary report of clinical use of GM-CSF in conjunction with amphotericin B that has given encouraging results (Torres, C. et al. 1993).

1.15.6 Adjunctive measures

Apart from antifungal drug therapy, adjunctive measures may also be appropriate. Wherever possible early treatment of raised intracranial pressure (ICP) is probably important. This can be achieved by repeated lumbar puncture, use of acetazolamide, shunting and nerve sheath fenestration as available, and may prevent blindness (Lipson et al. 1989; Denning et al. 1991a; Johnston et al. 1992). It has been suggested that the low mortality rate in one study (5.5% in the first two weeks and 3.9% in the next eight weeks), as compared with other large, randomized trials, was helped by such careful management of elevated intracranial pressure (van der Horst et al. 1997). This serious complication of cryptococcal meningitis occurs not only in patients with AIDS, but also in immunocompetent individuals. The place of short-term steroid therapy in reducing ICP, inflammation and oedema is uncertain (Rex et al. 1993; Denning et al. 1991b). There is no clear evidence that surgical removal of cerebral cryptococcal mass lesions is required.

For infection outside the central nervous system there is no standardised therapy, although depending on site and degree of immunosuppression, amphotericin B is usually included in the regime prescribed, except in patients with pulmonary cryptococcosis alone, where in the immunocompetent careful observation is a reasonable course to follow.

1.15.7 Outcome

The outcome of infection reflects the ease with which any underlying disease can be controlled. In a retrospective study of patients with AIDS and neoplastic disease there was a median survivial of 9 months and 2 months respectively (White et al. 1992). In all areas the mortality of cryptococcal meningoencephalitis is high, ranging from 9.4 to 60% (Diamond and Bennett, 1974; Slobodniuk and Naraqi, 1980; Fisher et al. 1993; Gould and Gould, 1985; Tay et al. 1972; Tjia et al. 1985; Spickard et al. 1963; Kerkering et al. 1981; van der Horst et al. 1997) perhaps higher in the tropics due to later presentation, lack of resources for optimal management or possibly more common var. *gattii* infection. Hydrocephalus contributes to this mortality, with the reported incidence varying from 6.6% to 74% (Diamond and Bennett, 1974; Diamond, 1995; Gould and Gould, 1985; Tjia et al. 1985; De Wytt et al. 1982). Even amongst survivors the morbidity is considerable with visual loss, chronic brain syndrome, motor impairment and unresolved cranial nerve palsies (De Wytt et al. 1982; Diamond and Bennett, 1974).

1.15.8 Prevention

There is no evidence that occupational exposure to a particular potential source leads to infection, though similar isolates have been found in both patients and the environment (Currie et al. 1994). It would seem reasonable that immunosuppressed patients in particular should avoid undue exposure to avian dropppings, but given the apparent rarity of var. *gattii* infection in AIDS and other immunosuppressed patients, there is no evidence to support a particular recommendation for these patients to avoid eucalyptus trees. It might be that if var. *gattii* is found consistently in the environment during flowering of such trees, it would generally be advisable to avoid exposure at such certain times of the year.

Human immunisation with *C. neoformans* GXM conjugated to tetanus toxoid has produced antibody that improved survival in mouse cryptococcosis when used for passive immunization (Devi et al. 1991; Williamson et al. 1993). In the future it may be possible to develop a vaccine to protect those with HIV infection in particular. As antifungals are used in AIDS patients for other indications, the incidence of cryptococcal meningitis may fall, as has been found in patients treated with fluconazole for mucocutaneous candidiasis (Powderly et al. 1995; Manfredi et al. 1997b).

1.16 Papua New Guinea

1.16.1 Introduction

Papua New Guinea (PNG) is comprised of a diverse group of islands lying between 1 and 12 degrees south of the Equator, lying at its closest point some 160km north of Australia across the Torres Strait. The main land mass of New Guinea is divided into Irian Jaya (under Indonesian control) and PNG. The country is spread over an area 1000 kms by 1500 kms, much of which is water, being bounded on the north by the Bismarck Sea, on the west by the Solomon Sea and on the south by the Coral Sea (Plate 1.3)

The main islands rise to 2,000 metres whilst on the main land mass of PNG there is a massive central spine of mountains, the Owen Stanley Ranges, which rise to 4,509 m at their highest point within PNG, Mount Wilhem, which at times is snow capped. The geographical situation of the county, with the variations in altitude, contribute to a wide range of climatic conditions, which allow a diverse flora and fauna to flourish. Included in this are thirty eight species of bird of paradise and two thirds of the world's orchids.

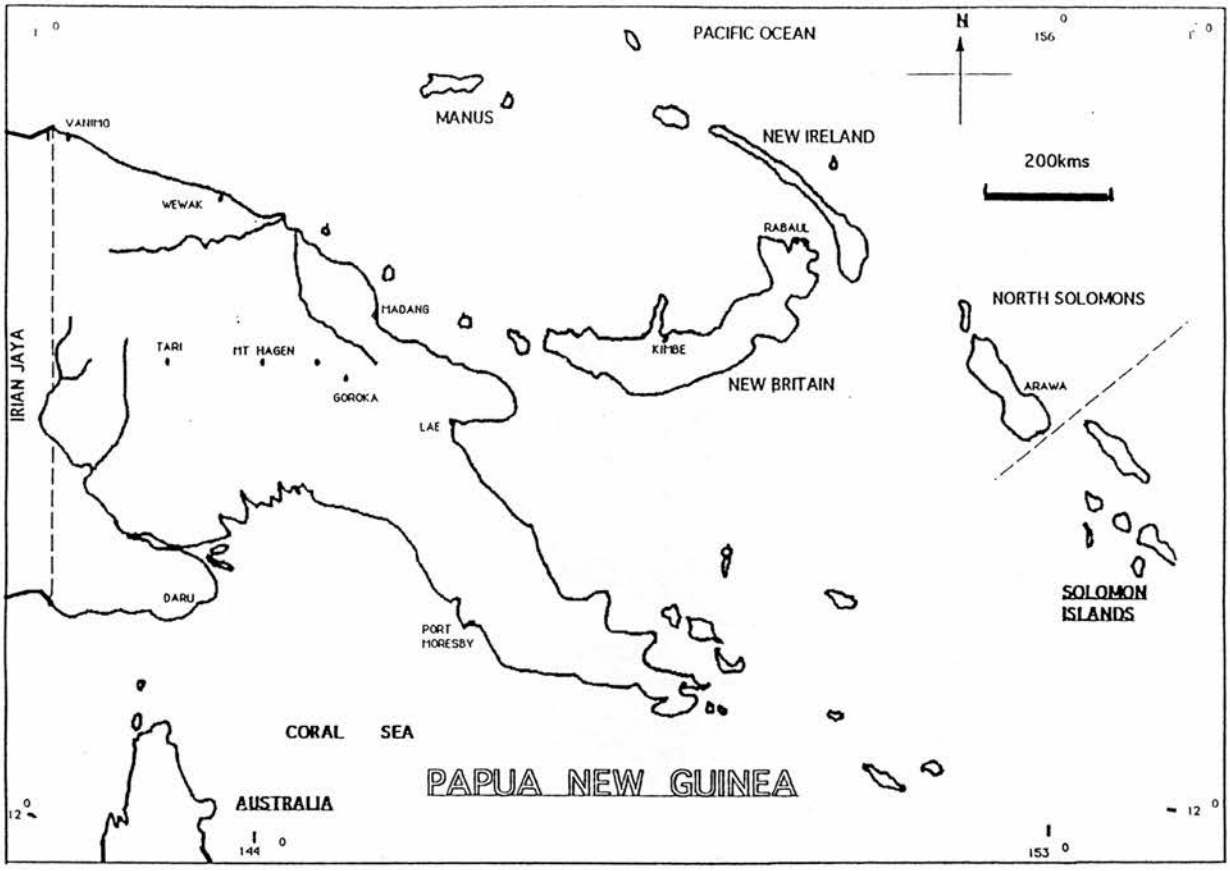

Plate 1.3 Map of Papua New Guinea

The population is 3,529,538 growing at a rate of 2.4% per year (1990 Census), which speaks over 700 languages, 45% of all the languages in the world (Anonymous, 1993a). The principle *lingua franca* is Pidgin English, with English being in use in the urban centres and for international communications. There are strong ties between those from the same village who therefore speak the same language and are known as 'wantoks' ("one talk!"). Owing to the difficulty of travel and communication, many villages have only had contact with the outside world for a few decades; there are still occasional reports of a 'new' group being discovered by those 'on patrol'. The people are of Melanesian origin, and traditionally subsist by hunting, fishing and gardening with some local and coastal trade. In some fertile valleys in the mountainous areas there have been attempts to introduce cash crops such as coffee and in the recent past coastal areas exported copra. There is now a burgeoning bureaucracy in the main centres and exploitation of natural resources such as timber, gas, gold, copper and platinum, which is resulting in literally explosive changes.

Port Moresby is the Capital of PNG and lies within the National Capital District (NCD), bounded by the Coral Sea and Central Province (CP) in the southern, Papuan region of the country. PNG became independant in 1974, having been an Australian protectorate. It has a democratically elected parliament in Port Moresby, with local governments in each Province. The head of state is Queen Elizabeth II of Great Britain ('Missis Kwin') who is highly regarded. Rather impractically for a capital, Port Moresby has no road links outwith NCD and CP, requiring travel by air or sea to reach other provinces with any ease. Within the Capital, crime flourishes,

related to unemployed migrants in search of cash earnings and the excesses of alcohol. This is relatively unencumbered by effective policing. Many 'expatriates', especially those in High Commissions and Embassies are fenced within mini-fortresses guarded by alarms, dogs and barbed wire. Some of the 'rural migrants' live in settlements in and around Port Moresby without access to adequate housing and water facilities. As a result communicable disease such as tuberculosis, typhoid and diarrhoeal disease are endemic in these areas.

The University of Papua New Guinea (UPNG) is based in Port Moresby and includes a Faculty of Medicine at the teaching hospital, Port Moresby General Hospital (PMGH). Students from all over PNG and the western Pacific attend this Faculty which has National Papua New Guinean and expatriate staff. PMGH is a tertiary referral hospital for PNG with 600 beds serving local populations of 193,242 in the NCD and 140,584 in CP. There are 12,000 admissions per annum, with microbiological, haematology and pathology laboratories on site. In addition to communicable diseases, conditions such as cardiovascular disease and diabetes mellitus are becoming more prominent reflecting changing life-styles, particularly in the urban centres. Most health care in PNG is provided by Aid Post workers and Health Extension Officers with doctors in the major centres and mission/volunteer hospitals. Traditional healers are also frequently consulted as many afflictions are thought to have social and spiritual roots. Occasionally patients 'abscond' from hospital or default from clinics to attend these healers.

1.16.2 Cryptococcal meningitis in Papua New Guinea

The pattern of cryptococcosis in PNG is largely consistent with that already outlined. The first recognition of cryptococcosis in PNG was by Backhouse, described by Cox and Tolhurst (Cox and Tolhurst, 1946). Here a "torula collection" in the lung is mentioned at post mortem in a New Guinean patient. Meningitis was first reported in a Melanesian male from Morobe Province, but who had been working in East New Britain (Champness and Clezy, 1962). His initial complaints were respiratory, but he then developed signs of meningitis and was found to have a lymphocytic CSF with low sugar levels. Despite subsequent anti-tuberculous therapy he died and brain sections examined at the School of Tropical Medicine, Sydney, showed cryptococcal forms with mucicarmine staining capsules, consistent with *Cryptococcus neoformans*. At Port Moresby General Hospital two patients with cryptococcal meningitis were reported out of 2000 consecutive admissions in 1965 and then in a review of deep mycoses in Australia and New Guinea in 1970, a case reported by Hickie and Walker in 1964 is mentioned (Campbell and Arthur, 1964; Frey and Durie, 1970). The next report in the literature is of a 30 year old male from Madang Province also working in East New Britain (Kariks, 1967). He presented with chronic headache, papilloedema and a solitary chest Xray lesion. Lumbar puncture showed a raised CSF pressure of 30cm and lymphocytosis, but Gram and Ziehl-Neelsen stains were negative. He was treated for tuberculosis but died shortly afterwards. Autopsy demonstrated cryptoccoci in the brain and lung (Kariks, 1967).

In 1980 Slobodnuik and Naraqi described the first series from Papua New Guinea of 13 adult cases occurring between 1972 and 1978 (Slobodniuk and Naraqi, 1980). These patients with cryptococcal meningitis were mainly young adults all of whom were apparently immunocompetent. This study showed that cryptococcal meningitis was the commonest form of chronic meningitis in adults, tuberculous meningitis being second. Mortality was 60% and attributed to frequent misdiagnosis of tuberculous meningitis in an area with a high incidence of tuberculosis.

A report on neuropsychiatric aspects of cryptococcal meningitis in PNG was made in 1980 indicating its presentation as an acute organic psychosis (Andrew and Burton-Bradley, 1980). In 1987 129 cases diagnosed as having cryptococcal meningitis between 1978-87 were noted (Temu et al. 1987). This was largely on the basis of laboratory data, including results of antigen testing on sera received from other hospitals in PNG. The apparent increase in incidence of cryptococcosis probably reflects an increasing awareness of the disease, and the availability of cryptococcal latex antigen testing of CSF, serum and urine. Up to this point no CSF isolates from PNG had been biotyped but by 1984 it was recognised that the distribution of the two biotypes of *C. neoformans* varied, with var. *gattii* predominating in tropical and subtropical regions and var. *neoformans* being found in all regions (Kwon-Chung and Bennett, 1984). When six biotyped isolates from apparently immunocompetent individuals presenting at PMGH were reported in 1990, five were shown to be var. *gattii* and one var. *neoformans*, suggesting that this geographical distribution holds true for PNG (Currie et al. 1990).

There is also evidence for non human sources of cryptococci in PNG. *C. neoformans* has been reported as the cause of mastitis in cattle (Anonymous, 1965). Then in 1984 pulmonary cryptococcois in a rat in Rabaul, East New Britain was described (Scrimgeour and Purohit, 1984). Mucicarmine and immunofluorsence stains confirmed the cause of cystic pulmonary lesions to be *C. neoformans*. They sugggested that the rat may have a role in transmission of cryptococcosis to humans as it sometimes nests in the rooves of houses. The only confirmed isolation from the environment was again in Rabaul when a soil suspension was injected intraperitoneally into a mouse and the *C. neoformans* identified in the mouse (Frey and Durie, 1964). This was prior to the differentiation of the two varieties of *C. neoformans.*

Since the studies cited above, further work to be described in this thesis has been carried out in PNG as well as Edinburgh. The aims of the studies described in this thesis were to:

1) Identify potential environmental sources of *C. neoformans* in PNG.

2) Attempt to isolate the yeast from such sources.

3) Investigate the epidemiology of *C. neoformans* meningitis in PNG.

4) Describe the clinical features of a prospectively observed series of cryptococcal meningitis cases in PNG.

5) Study human-neutrophil cryptococcal interactions.

6) Highlight the need for appropriate culture in addition to microscopy and latex antigen testing of CSF in order to diagnose cryptococcoal meningitis. An apparently acapsulate isolate was studied in part using the human neutrophil-cryptococcal assay.

These studies were carried out in PNG and Edinburgh and are described in this thesis. They have formed the basis for further observations in PNG subsequently made by Seaton and other colleagues, which will be alluded to in the discussions.

CHAPTER 2

Potential environmental sources of *Cryptococcus neoformans* in PNG: eucalypts, mammals and birds

2.1 Introduction

2.1.1 Natural history

The flora and fauna of PNG are diverse. The dominant influences come from Australia, although much has not been assessed by systematic specialists. As discussed by Slater, there are no large mammal predators and the largest indigenous mammals are marsupials (Slater, 1959). Differences from related species elsewhere have arisen over long periods of geographical isolation. For example the flying phalanger (*Petaurus breviceps papuana*), a type of possum, agile wallaby (*Protemnodon agitis papuana*) and tree kangaroo (*Dendrologus goodfellowi shanmayeriari*) are endemic to PNG, whereas the airborne spectacled fruit bat (*Pteropus conspicillatus*) is found both in Australia and New Guinea.

The scope of birds is less constrained than that of land-based animals, but there are none-the-less unique adaptations in those with reduced range extension imposed by exacting environmental limitations. These limitations include tolerance to climatic conditions, altitude and vegetation and the degree of efficiency and power of flight. Within such constraints the flightless Cassowaries (*Casuarius* spp), and spectacular crowned pigeons (*Goura* spp) have evolved.

The reptilian populations again reflect the proximity to Australia in the sub-specific variations including the taipan (*Oxyranus scutellanus canni*), death adder (*Acanthopis antarticus*), New Guinea Crocodile (*Crocodilus novaeguineae*) and Estuarine Crocodile (*C. porosus*). The range of the *Viperidae* family stops west of

New Guinea, which is the 'citadel' of the front fanged venomous snakes of the family *Elapidae*.

The vegetation of the island of New Guinea is also complex and varied. In lowland areas there is tropical jungle, inundated with large quantities of water, and adjacent well drained areas with lower rainfall, resulting in savannah with sparse trees and shrubs. At higher altitudes, from 1000 to 2200 metres there is sub tropical forest, with temperate and then alpine areas in the highest ranges. The genus *Eucalyptus* can thrive from sea-level to temperate areas, depending on the species, soil, position and amount of precipitation. Typical savannah near Port Moresby is shown in Plate 2.1.

2.1.2 Ecology of *Cryptococcus neoformans*

C. neoformans var. *neoformans* has been found in close association with avian, particularly pigeon, droppings and soil associated with such droppings, since Emmons first report in 1951 (Emmons, 1951; Emmons, 1955). Since this time a similar link has been confirmed in many parts of the world. Staib showed that *C. neoformans* utilised creatinine as a nitrogen source, but other species of *Cryptococcus* did not (Staib, 1962b). Var. *gattii*, which does not occur in pigeon droppings actually utilises creatinine better as a nitrogen source than var. *neoformans* (Bennett et al. 1978), so it would seem unlikely that creatinine use is important in determining the ecological differences between the two varieties.

Plate 2.1 Typical savannah outside Port Moresby

Whether the infectious propagule of *C. neoformans* is the asexual or sexual form is uncertain. The basidiospores of *Filobasidiella neoformans* are dry, may be present in air and are small enough (1.8 - 3μm diameter) to be deposited in the alveoli. Desiccated yeasts may also be small enough to reach the alveoli following inhalation. Perhaps the reservoir for this form is on plant matter rather than in avian droppings. This was suggested by Ellis and Pfeiffer following their discovery of var. *gattii* in association with flowering *Eucalyptus camaldulensis* (Ellis and Pfeiffer, 1990a; Ellis and Pfeiffer, 1990b). They proposed that the distribution of var. *gattii* disease reflects that of the natural host of this fungus, initially suggesting that the countries to which *E. camaldulensis* had been exported were those in which *C. neoformans* var. *gattii* could be found causing human meningitis. They pointed out that the koala, which feeds on eucalypts develops cryptococcosis (Bollinger and Finckh, 1962) and they were able to isolate this variety from koalas (Ellis and Pfeiffer, 1990a). Currie et al. suggested that *E. camaldulensis* was not the source of var. *gattii* in PNG as it is not indigenous here (Currie et al. 1990). As discussed already, cryptococci had previously been found causing bovine mastitis, rat pulmonary infection and in soil within PNG.

Against this background investigation of potential environmental sources of *C. neoformans* was begun. The aim was to identify potential sources of *C. neoformans*, particularly var. *gattii* in the environment in PNG. Specifically the hypothesis that *Eucalyptus camaldulensis* or a similar plant source is associated with *C. neoformans* var. *gattii* in PNG was considered. Birds and mammals would also be identified that

may be linked as sources, reservoirs or means of distribution of this fungus from potential plant hosts.

2.2 Methods

Sources of information

There are University and National Libraries, both in Port Moresby. These were helpful sources of reference books on flora and fauna of PNG. In addition at the main UPNG campus, Waigani, there is a Herbarium adjacent to the Department of Biology. This contains plant material and documents with species identification, date and location gathered over many years. The staff in these departments were interested and helpful in guiding me, particularly Dr Ian Burrows Head of Biology, Dr Simon Saulei and Mr Max Kuduk. They suggested further discussions with Mr Neville Howcroft of the Forestry Research Institute who had carried out fieldwork in many parts of PNG. He was able to give me details of *Eucalyptus* species derived samples held at the Forestry Research Institute Herbarium in Lae, Morobe Province. A further source of information was identified in the library of the Department of Forests in Port Moresby. Latterly information has been obtained from purchased published texts and those in the Darwin Library of Edinburgh University.

2.3 Results

2.3.1 The Genus *Eucalyptus*

This genus was first described and named by the French botanist L'Heritier in 1788. He studied specimens probably collected in Tasmania on Cook's third voyage in 1777. Evidence from fossil pollen suggests that ancestral eucalypts existed thirty million years ago (Costermans, 1994).

Now over 600 species are recognised (authorities vary in their interpretation of 'species'). The genus *Eucalyptus* is the largest in the family Myrtacae and indigenous species are largely limited to Australia and nearby islands. Like other members of Myrtacae they have hard-textured leaves with oil glands and woody fruit capsules. Most are trees, though some, mainly the mallees, are more shrub like. They are more common in coastal areas with regular rainfall, but inland are associated with periodic watercourses and subsoil moisture. Some species, notably the River Red Gum (*Eucalyptus camaldulensis*) and Blue Gum (*Eucalyptus tereticornis*) have been widely exported (Jacobs, 1979).

In common with other plant groups originating in Australia eucalpyts have an evolutionary relationship with fire (Jacobs, 1979; Costermans, 1994). They constantly drop dry leaves and bark flakes, have volatile, flammable oils in the leaves and combustable wood. Their mechanisms of surviving the effects of fire vary between species. This depends on insulating properties of the bark, the ability to produce emergency epicormic buds under the bark, which become new branches

after the crown of the tree has been burnt out. Also the mallees in particular have lignotubers which are swellings at the base of the trunk with food reserves and dormant growth buds which can produce new stems from ground level. Some species are killed by fire, but the heat causes fruit capsules to open and drop seeds onto burnt ground, allowing regeneration.

2.3.1.2 Classification and Identification

The genus *Eucalyptus* can be divided into seven subgenera *Blakella*, *Corymbia* (bloodwoods), *Eudesmia, Gaubaea, Idiogenes*, *Monoclyptus* (peppermints, sallees, stringybarks, ashes), and *Symphomyrtus* (mahoganies, boxwoods, mallees, gums ironwoods) (Pryor and Johnson, 1971). These are subdivided into sections, series, subseries, superspecies, species and subspecies. The 'red gums', section *Exsertaria*, are part of the subgenus *Symphomyrtus* (Pryor and Johnson, 1971). These cannot survive where ground freezes in winter. Species are distinguished on the basis of numerous characteristics, notably bark type, leaves (juvenile and adult), buds, anthers, flowers and fruits. The size of growth, location and geographical distribution can also give an indication as to the species. Hybridisation may occur where both parents are or have been in the same area with overlapping flowering periods and are in the same subgenus. This complicates species idenfication considerably, even for experts.

2.3.1.3 Eucalypts occurring naturally in PNG

There are at least 6 indigenous *Eucalyptus* species in PNG as shown in Table 2.1. (Jacobs, 1979; Boland, 1957; Saulei and Howcroft personal communication). In the National Capital District and Central Province, the predominant species are *E. papuana*, *E. confertiflora* and *E. alba* (Heyligers, 1982). These are shown in Plates 2.2 and 2.3. These species dominate the open grassy woodlands in Central Province, as well as thriving in Port Moresby itself. The canopy may be up to 16 metres in height. The soils in this region are neutral or mildly acidic; eucalypts are sparser on alkaline soils. *E. alba* and *E. confertiflora* exhibit facultative deciduousness, with the severity of the dry season determining how much foliage is shed. They do not regenerate under forest conditions and need fire such as may occur as a result of thunderstorms to trigger rapid regeneration.

E. tereticornis occurs at altitudes up to 6,000 feet in the Sogeri Plateau, in the Astrolabe Range and Hombrum's Bluff, inland from Port Moresby, as well as being present at sea level, particularly south eastwards in Rigo and Abau in Central Province. Elsewhere in PNG it is found in Northern Province, Western Province around Daru and the Transfly areas and in eastern Australia from Queensland to Victoria (Boland, 1957). This species can be wind pollinated, unlike many others.

Table 2.1 ***Eucalyptus*** **species indigenous to PNG**

Species/Sites	Common Name	Province	Sources of data
E. confertiflora	cabbage gum	NCD, Central, Western	IFL, FAO, FRI, Hey, UPNG
E. papuana	ghost gum	NCD, Central, Western	IFL, FAO, Hey, UPNG
E. alba	white gum	NCD, Central	IFL, FAO, Hey, UPNG
E. tereticornis	forest red gum, Queensland blue gum	Central, Western, Northern	IFL, FAO, UPNG, FRI
E. deglupta	kamarere	Central, Northern , Sepik, Morobe, Milne Bay, New Britain, New Ireland	IFL, FAO, UPNG, FRI
E. brassiana		Western	FRI, FAO

IFL - own observations, FAO - (Jacobs, 1979), FRI - Forestry Research Institute Herbarium, Lae, Hey - (Heyligers, 1982), UPNG -University of Papua New Guinea Herbarium specimens and documents. NCD - National Capital District.

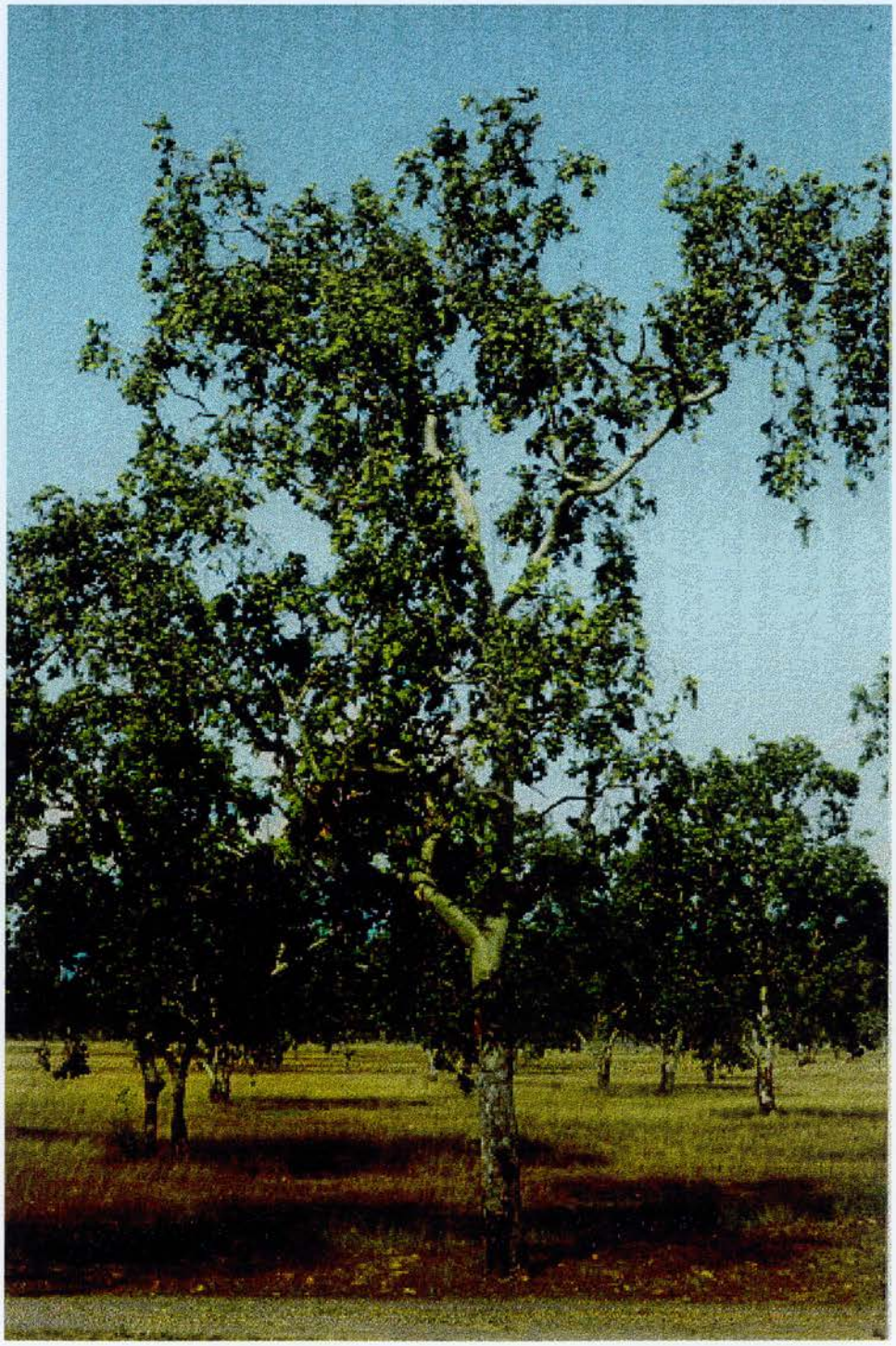

Plate 2.2 ***Eucalyptus confertiflora*** **at the University of Papua New Guinea**

Plate 2.3 ***Eucalyptus alba*** **at the University of Papua New Guinea**

E. deglupta is also found in the eastern parts of Central Province, but is more commonly found in indigenous sites in the Northern Province, around Oro Bay, Popondetta. Reliable observers have reported this species at several other sites in PNG namely Vanimo Sepik, Morobe, Northern and Milne Bay New Britain (Documents at UPNG Herbarium).

There are many herbarium specimens of *E. brassiana* at the Forestry Research Instutue in Lae collected at Daru in Western Province.

It is of interest to note that there are indigenous species of the red gum group exsertaria, closely related to *E. camaldulensis.* These are *E. tereticornis*, *E. brassiana, E. alba* and *E. papuana.*

There is considerable uncertainty over identification or indigeneity of further species, *E. polycarpa* (bloodwood) in Western Province, and *E. platyphylla* to the east and west of Port Morsby (UPNG Herbarium documents).

2.3.1.4 Introduced eucalypts and plantations

Skelton, who summarised information from many sources, including the verbal history of experienced foresters, states that there was little 'serious reforestation' prior to 1950 in PNG (Skelton, 1981). The earliest plantation documented is of teak in Madang, which is of doubtful quality and may now be 'contaminated' by shrapnel. In the early 1950's the natural state of forestry was estimated by aerial photography and sampling by ground surveyors. Local and national government

forestry departments tried to establish a number of plantations for experimental and commercial exploitation. PNG is a 'new' country in developmental and geological terms leading to high rates of soil erosion because of steep slopes and ridges. As a result the rivers carry high silt loads. The upshot of this and periodic fires is that there are fewer large trees than are seen in Africa and Asia.

Skelton's observations (relating to both government and privately owned plantations and records) and information from the First Papua New Guinea Silvicultural Research Conference in 1968 are summarised in Table 2.2. I found no information on any similar subsequent conference.

Table 2.2 Eucalypt plantations in PNG

PROVINCE (site)	START DATE	*EUCALYPTUS* SPECIES	SUCCESS (of plantation)
Morobe (mainly Bulolo)	1951	*alba acmenoides camaldulensis citriodora deglupta gigantea grandis micorocorys muelleriana pilularis robusta saligna scabra sideroxylon torelliana tereticornis torelliana alba*	no
East New Britain	1948	*deglupta*	yes
Central (mainly Brown River)	1955	*citriodora deglupta grandis camaldulensis microtheca miniata nisophila paniculata citriodora torelliana tereticornis brassiana*	no
Eastern Highlands (Lapegu)	1963	*deglupta grandis camaldulensis alba*	no
Eastern Highlands (Kainantu, Bena Bena)	1976	*stjohnii robusta saligna regnans rubida dalrympleana nitens goniocalyx obliqua vinialis maidenii globulus grandis torreliana*	no
Eastern Highlands (Goroka)	1959	*alba camaldulensis citriodora cladocalyx cloewiana dalrypmleana decasiana (urophylla) fastigantea globulus grandis goniocalyx leucoxylon maculata maidenii marginata mulleriana nitens obliqua pnalicuate pauciflora pilularis frensns resinfiera robusta rubida saligna scabia siberiana sideroxylon stellata tereticornis torelliana viminalis*	unknown
Western Highlands (Hagen)	1958	*alba camaldulensis citriodora cladocalyx cloeziana gigantea globulus grandis maculata maidenii microcorys ornata pilularis robusta saligna scabra siveriana sideroxylonstellulata tereticornis viminalis*	unknown
Western Highlands (Whaggi swamps)	1972	*robusta grandis*	yes, but land problems
Madang	1969	*deglupta brassiana deglupta tereticornis robusta urophylla*	poor mixed
East Sepik	1964	*tereticornis grandis deglupta*	no
West Sepik	1969	*deglupta torelliana tereticornis robusta brassiana saligna*	no
Southern Highlands	1982	*grandis robusta stjohnii*	unknown
Enga	1982	*grandis robusta stjohnii* & other sp not identified	unknown
West New Britain	1967	*deglupta*	no
Northern (Oro)	1975	*tereticornis deglupta*	not stated

Data summarised from (Skelton, 1981; Anonymous, 1968).

There have been additional commerical schemes, but information on these is not available in any collated form. Since 1950 *E. camaldulensis* has been planted in trials at Brown River, Central Province, Lapego and Mount Hagen, Western Highlands Province, Goroka, Eastern Highlands Province, Bulolo, Morobe Province) (Skelton, 1981), and at sites in East and West Sepik and Madang Provinces (Saulei, personal communication). Virtually all the known plantations of *E. camaldulensis* have failed, with poor tree growth and death in many instances (Saulei and Howcroft, personal communication). Occasional trees do survive - for example, there are a few specimens on the Bulolo golf course in Morobe Province. The main reason for failure of many plantations has been land ownership disputes between local villagers and government and plantation companies, leading to prevention of further forestry access and poor maintenance of plantations. Government funding of forestry has dwindled since the early days of Australian administration, contributing to plantation failure. Fire too has destroyed other plantations. Thus the success rate has been low for reasons other than those of purely climatic or geographical origin.

2.3.2 Mammals

2.3.2.1 Introduction

Flannery's monograph 'Mammals of New Guinea' provides the most up to date overview of this topic, drawing together many communications and personal observations with a review of the literature (Flannery, 1990).

Knowledge of the mammals of New Guinea has been accumulated only over the past 100 years or so, important initial contributors being Luigi D'Albertis, Michael Thomas and George Tate. The former was an explorer and field scientist who lead expeditions in the 1870's, collecting for the Museo Civico di Storia Naturale in Genoa, Italy. Based at the British Museum, Thomas described material collected by others, including D'Albertis, but never visited his subjects' countries of origin. Tate was both an active systematist and field worker, employed by the American Museum of Natural History during the 1920's. Since the work of these men, museums in Hawai, Leyden, Berlin, Paris, Canberra, Sydney and Port Morseby have all researched and collected New Guinean mammals.

2.3.2.2 Indigenous Mammals

Today at least 187 indigenous, 13 introduced and 11 extinct species of mammal have been identified from the island of New Guinea (Flannery, 1990). A summary of indigenous mammals is given in Table 2.3 and examples illustrated in Plates 2.5 and 2.6. Those mammal species where mention in the literature of feeding on eucalypts or related species is made are particularly highlighted. In many instances little is known of their diet, natural history and range, even by the local peoples.

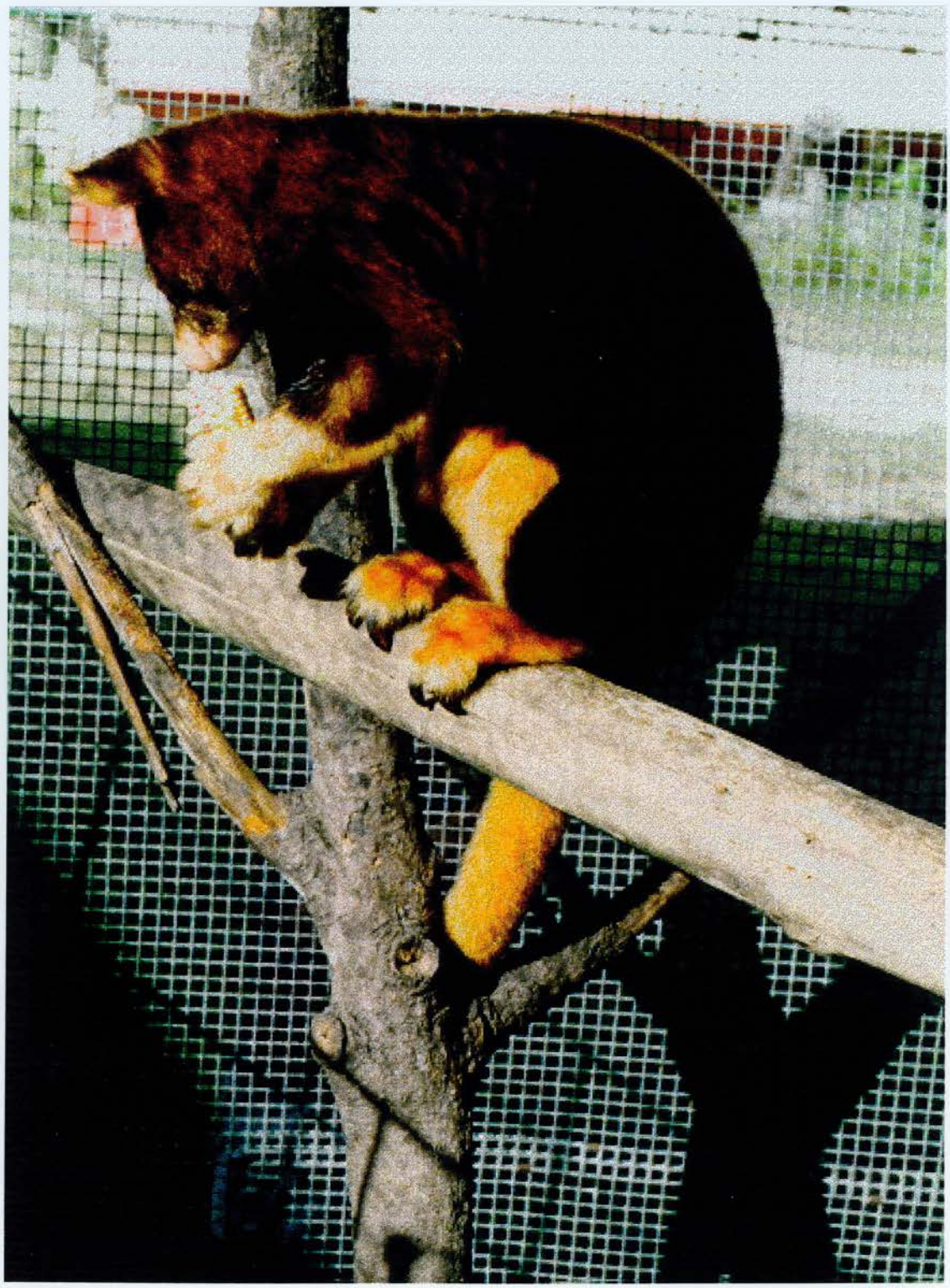

Plate 2.4 **Goodfellow's tree kangaroo (*Dendrolagus goodfellowi*)**

Plate 2.5 Spotted cuscus (*Spilocusus maculatus*)

Table 2.3 Indigenous mammals of New Guinea

FAMILY	Genera	Species	COMMENTS
Tachygloissidae	2	2	Both echidna species are edentulous large-brained spiny insectvorous monotremes.
Dasyuridae	8	14	Includes *Phascalosorex dorsalis* (narrow striped dasyure)
Peroyctidae	3	8	Bandicoots and echmiperas
Peramelidae	1	1	*Isoodon macrourus* (Northern brown bandicoot)
Macropodidae	5	15	Includes tree-kangaroos (*Dendrolagus dorianus, D. goodfellowi, D, matschiei, D. spadix and D. urisnus*) which eat fruit and figs, wallabies (*Dorcopsulus* spp) and agile wallaby (*Macropus agilis)* feed on *Eucalyptus* sp. leaves and figs, found near Port Moresby (Merchant, 1983).
Phalangeridae	3	8	Includes *Phalanger carmelitae* (mountain cuscus) - eats casuarina leaves and pandanus fruits, *P. sericeus* (silky cuscus) - eats young foliage of myrtaceous plants, including *Eucalyptus* sp.
Acrobatideae	1	1	*Disoechurus pennatus* (feather tailed possum) eats fruit and insects.
Burramyeliae	1	1	*Cerartetus caudus* (long tailed pygmy possum).
Petauridae	2	5	*Oposum layang biasa* (sugar glider) known to eat eucalypt sap in Australia (Henry and Suckling, 1985).
Pseudocheiridae	2	8	*Pseudocheirus forbesi* (painted ringtail) found in montane forests.
Muridae	22	57	Rats and mice.
Pteropodidae	7	19	Bats including 'flying foxes'. *Pteropus scapulatus* (little red flying fox) main food *Eucalyptus* sp. blossoms. *P.alecto* (black flying fox) preferred food includes *Eucalyptus* sp. blossom (Hall, 1983). *P. neohibernicus* (greater flying fox) largest New Guinean bat feeds on fruits; -widespread below 1000m. Also 'blossom bats': *Syconycteris hoggit, S. australis, Macroglossus minimus*.
Emballonuridae	3	9	Mainly cave roosting bats. Many spread to Moluccas, Asia and Australia
Hipposideridae	2	10	Mainly cave roosting bats
Rhinophidae	1	4	'Horse-shoe' bats
Vespertilionidae	10	21	Obligatory cave roosting bats. Some widespread: Japan and New Caledonia to Mediterranean eg *Miniopterus schreibersi*
Molossidae	4	6	Fly above forest canopy so difficult to catch and study. Little known.

Numbers of genera and species in each family shown. Unreferenced data compiled from (Flannery 1996).

2.3.2.3 Introduced mammals

A number of mammals have been introduced to New Guinea, generally linked to human inhabitation (Flannery, 1990). *Homo sapiens* has been a component of the fauna for at least 40,000 years (Groube et al. 1986). In the latter part of this time there has been contact with peoples from surrounding landmasses, and possibly several waves of imigration. The feral pig, *Sus scofa* is hunted widely and relied upon to impregnate domestic sows. Pigs can damage forest understorey and so affect the habitat of other animal and plant species. Dogs, in the form of *Canis familiaris*, constitute feral populations which do not bark, yet howl! Various forms of rat have been introduced in conjunction with *Homo sapiens*, including *Rattus rattus* which is mainly found in the lowlands near human habitation. Another introduced murid species is *Mus musculus* which is a serious pest in coastal towns and villages as well as occurring in grasslands around Port Moresby and elsewhere. Three species of deer have been introduced. *Cervus timoriensis* (Rusa) arrived in 1928 brought by the Dutch (this species is now used as a source of meat). *Dama dama* (fallow deer) and *Axis axis* (Axis deer) were also introduced to the Madang area of PNG. Details of their introduction are uncertain. Finally feral cats (*Felis catus*) occur as a result of domestic cats 'going wild'. Little is known of their impact on local fauna.

2.3.3 Birds

The birds of New Guinea are also extremely diverse, there being some 570 endemic non-marine species, including 445 forest dwellers (Beehler, 1985). This diversity (on an island second only to Greenland in size) arises because of the large areas of forestation, proximity to the Australian landmass, and the islands linking to Asia. A further 40 seabird and 90 migrant species have been identified in New Guinea and associated islands. (Beehler et al. 1986). Approximately 71% of PNG is forested; 65% of the country is undisturbed rainforest and 20% man disturbed, with mountains climbing to 5,000m in Irian Jaya (the western half of the island of New Guinea). As in other aspects of flora and fauna, formal documentation of the bird species has only occurred over the last century through exploratory natural science expeditions. Groupings and species that feed on plant nectar and fruits and or may be found near human habitation are shown in Table 2.4. Rainbow lorikeets (example shown in Plate 2.7) frequently flew and roosted in trees within Port Moresby. In some villages near Port Moresby pigeons and cassowaries (Plate 2.8) are also kept as pets and sources of eggs or meat. At Laloata Island near the capital several species of bird and mammal are kept in captivity and birds for study are kept at the Christensen Research Institute near Madang.

Plate 2.6 **Lorikeet (*Trichoglossus haematodus*)**

Plate 2.7 Cockatoo

Table 2.4 Examples of bird groups feeding on sap/nectar and/or found near human habitation

Group/Family	Species	Examples
Parrots, Lories and Cockatoos: *Psittacidae*	46	*Electus roratus* (Electus parrot), common near human habitation, *Probosciger aterrimus* (Palm cockatoo), *Trichoglossus haematodus* (rainbow lorikeet), feed on nectar, fruit, seeds, garden bird.
Bower birds: *Ptilonorhynchidae*	11	*Chlamydera cerviniventris* (Fawn-breasted bowerbird), savannah and town habitats, *Archboldia papuensis sanfordi* (Sanford's Bowerbird), rare.
Birds of paradise: *Paradisaeidae*	38	*Macgregoria pulchra* (MacGregor's Bird of Paradise) *Paradisaea rudolphi* (Blue Bird of Paradise) *Epimachus fastuosus* (Black Sicklebill), *Ptilris magnifica* (Magnificent Riflebird) - lowland species.
Cassowaries: *Casuariide*	3	*C. casuarius* (Southern cassowary)
Drongos: *Dicruridae*	2	*Dicrurus hottentottus* (Spangled Drongo), found near gardens
Honeyeaters: *Meliphagidae*	65	*Melistes megarhynchus* (Longbilled Honeyeater)
Megapodes: *Megapodiidae*	7	*Megapodius freycinet* (Common scrub fowl) lowland forest, lays eggs in mounds of humus
Pigeons and Doves: *Columbidae*	45	*Columbia livia* (Rock Pigeon), *Goura cristata* (Common Crowned Pigeon), *G. scheepmakeri* (Scheepmaker's Crowned Pigeon), *G. victoria* (Victoria Crowned Pigeon), *Ducula zoeae* (Zoe Capped Fruit Dove), *Ptilinopus pulchellus* (Crimson Capped Fruit Dove)

(Compiled from Beehler, 1985; Beehler et al. 1986; Pryor and Johnson, 1971)

Many of the rarer species' numbers are at risk of falling due to factors such as large size and ease of human disruption, small geographical ranges, being hunted for human food or ceremonial plumes and being colonisers of rare habitats. The birds of paradise are national emblems of PNG and regional emblems of Irian Jaya, reflecting their cultural significance and hence the value of their plumes.

2.4 Discussion

It would appear that despite the attempted importation and planting of many *Eucalpytus* species, their success in plantations has not been good (Saulei and Howcroft, personal communication; Skelton, 1981). In particular *E. camaldulensis*, although imported and planted at several sites, has not in general thrived (Howcroft, Saulei personal communication). Two cases of confirmed *C. neoformans* var. *gattii* meningitis have originated from Goroka, one from Bulolo, where some *E. camaldulensis* survive, and other non biotyped cases have originated from all the areas mentioned above (See Chapter 4). Therefore it is possible that although a few clinical cases of cryptococcal meningitis could be linked to this species of *Eucalyptus*, it is unlikely to account for the majority of cases in PNG.

At least 2 cases of cryptococcal meningitis including one with confirmed *C. neoformans* var. *gattii* have come from the Sogeri Plateau, as well as further cases in the Rigo and Abau areas of Central Province. *E. tereticornis* is found in all these areas. *E. brassiana* is prevalent in the Northern and Western Provinces of PNG as well as being widespread in areas of Northern Australia (Boland, 1957); cryptococcal meningitis occurs in all these areas. *E. alba, E. confertiflora* and *E. papuana* are commonly found in and around Port Moresby and Central Province, areas where var. *gattii* meningitis is prevalent.

It therefore seems possible that there are several *Eucalyptus* species associated with *C. neoformans* var. *gattii*. Since this study was initiated, Ellis and Pfeiffer have

reported associations with *E. tereticornis* in Queensland, *E. rudis, E. gomphocephala* and *blakelyi* in Western Australia (Pfeiffer and Ellis, 1992; Ellis et al. 1996; Pfeiffer and Ellis, 1997). All four of these species are related to *E. camaldulensis*, being of the red gum group *Exsertaria*, as are *E. alba* and *E. brassiana*, which are found in PNG (Pryor and Johnson, 1971; Boland, 1957).

C. neoformans var. *neoformans* is well recognised worldwide in association with pigeon droppings. There have been cases of meningitis caused by this var. in PNG. Pigeons such as the imperial pigeons (*Ducula* spp) and fruit doves (*Ptilinopus* spp), occur in PNG, and could be linked with this variety. (Coates 1985). Other birds such as rainbow lorikeets (*Trichoglossus haematodus*) are endemic in PNG and feed and roost on eucalypts near human habitation (own observations; Coates, 1985). In addition some pigeons and cassowaries were observed within villages as pets. They could play a role in *C. neoformans* transmission.

Mammals could also be implicated. Cryptoccocosis has been reported in a rat (*Rattus rattus*) in Rabaul, PNG, and in the koala (*Phascolarctos cinereus* - a member of the supercohort marsupialia), in Australia (Scrimgeour and Purohit, 1984; Bollinger and Finckh, 1962). *C. neoformans* var. *gattii* has been isolated from koalas, which feed predominantly on eucalypts (Ellis and Pfeiffer, 1990b). There are many mammals, including marsupials in PNG. These may be involved in transmission of cryptococcosis in this country. For example fruit bats such as the little red flying fox (*Pteropus scapulatus*) and black flying fox (*P. alecto*) feed on sap, nectar and flowers of eucalypts (Hall, 1983).

Whether the birds mentioned above and mammals other than the rat and koala are infected by a host tree remains to be seen. Further investigations described in Chapter 3 attempt to extend the understanding of the ecology of *C. neoformans*.

CHAPTER 3

Sampling of potential environmental sources of *Cryptococcus neoformans* in Papua New Guinea

3.1 Introduction

The yeast like fungus *Cryptococcus neoformans* is distributed world wide, and can be serotyped on the basis of its polysaccharide capsule, giving four serotypes, A,B,C,D and some which serotype as A/D or are untypeable. Biotyping can be used to differentiate *C. neoformans* varieties *gattii* and *neoformans*. Clinical isolates of *C. neoformans* var. *gattii* occur in tropical and subtropical regions predominantly in apparently immunocompetent rural dwellers, whereas *C. neoformans* var. *neoformans* is found also in temperate regions, particularly in immunocompromised urban dwellers. In 1989 *C. neoformans* var. *gattii* was consistently identified during and after flowering in association with the red river gum tree, *Eucalyptus camaldulensis*, in South Australia and in New South Wales (Ellis and Pfeiffer, 1990b). A similar association was confirmed in California (Pfeiffer and Ellis, 1991) and more recently in Italy and India (Montagna et al. 1996; Chakrabarti et al. 1997). In 1991 after the start of this study *C. neoformans* var. *gattii* was found in sheltered humus debris of *E. tereticornis* (Forest red gum) (Pfeiffer and Ellis, 1992).

Recognising that the distribution of *E. tereticornis* and *camaldulensis* even in Australia does not exactly mirror that of clinical human cases, Ellis and Pfeiffer have continued to investigate other potential eucalypt sources, reporting in 1996 that they had now found an association with *E. gomphocephala* (tuart) and *rudis* (flooded gum) in Western Australia (Ellis et al. 1996). Livestock had contracted var. *gattii* disease and when the local environment was investigated further *C. neoformans* var. *gattii* was isolated from wool on fencing beneath these species of *Eucalyptus*. *E. blakelyi* (Blakey's red gum) has also just been reported in association with var. *gattii* (Pfeiffer and Ellis, 1997). All these euclaypts are red gums, related to *Eucalyptus camaldulensis*.

Var. *gattii* has also been isolated from koalas, which feed on eucalypts, and on one occasion from bat droppings (Ellis and Pfeiffer, 1990a; Lazera et al. 1993). *C. neoformans* var. *neoformans* has been isolated from avian, particularly pigeon excreta in both temperate and tropical climates. In PNG *C. neoformans* has been isolated from soil near Rabaul and has caused bovine mastitis (Frey and Durie, 1964; Anonymous, 1965).

The national incidence in PNG of cryptococcal meningitis is at least 4.3 patients per million population per year (caused predominantly by *C. neoformans* var. *gattii*) and is estimated to be 33-42.8 patients per million population per year in the National Capital District (NCD) and Central Province (CP) (Chapter 4 and (Seaton, 1996)). Disease occurs predominantly in HIV negative patients with no clinical, haematological or biochemical evidence of immunosuppression (Chapter 5 and (Slobodniuk and Naraqi, 1980; Lalloo et al. 1994; Seaton, 1996)).

E. camaldulensis is not endemic to PNG. Since 1955 *E. camaldulensis* has been planted in trials at several sites in PNG (Skelton, 1981). Some trees survive from these trials, but in general they have not thrived, as suggested by the records and observations of Department of Forestry, Forestry Research Institute and University Foresters and Biologists (Chapter 2; Currie et al. 1990; Skelton, 1981). Many cases of cryptococcal meningitis originate in areas where no *E. camaldulensis* have been planted. There are no records of export of *E. rudis, E. gomphcephala* and *E. blakelyi* to PNG, nor are they endemic to this country. The predominant and endemic species around Port Moresby are *E. alba*, *E. papuana* and *E. confertiflora*, with *E. tereticornis* and *E. deglupta* elsewhere in Central Province. Some species hybridize which can make definite identification difficult.

Attempts were made to determine the habitat of *C. neoformans* by sampling eucalypts, other plant sources, mammal and avian droppings in a number of provinces of PNG.

3.2 Methods

Sampling from environmental sites in Port Moresby, National Capital District, Central Province, Western Province, Madang and Rabaul (East New Britain) was carried out during 1991-4. In particular dust, soil and vegetation in and around houses of patients infected with *C. neoformans* var. *gattii* were sampled. Included were any nearby eucalypts, taking care where possible to sample from hollowed out areas, stem junctions and sheltered areas around trees. Sampling was performed on plants that had flowers and those that were not in flower. There is great variation in the timing and duration of flowering of eucalypts, depending on the local microclimate, which varies dramatically within a few kilometres. Avian and mammalian droppings were also sampled.

Samples of 5 - 15g material were collected in sterile containers and shaken in 20 mls sterile water or 0.85% saline and allowed to stand for 10 - 15minutes. The 47 samples from mammals in Western Province were frozen at -20^{0}C until thawed, diluted and cultured 3 months later. Heavy loopfuls (40-80μL) from the resulting suspensions were streaked onto *Guizotia abyssinica* creatinine agar (GACA) (also known as bird seed agar) plates and incubated at ambient temperature (24-26^{0}C) for 7 days (Ellis and Pfeiffer, 1990b). Positive controls using clinical isolates to spike environmental samples were set up with each batch at a concentration of approximately 1 x 10^3 colony forming units per milliliter, and the brown colour effect (BCE) and typical mucoid morphology of *C. neoformans* confirmed. Approximately 1ml of 25 flowering eucalypt associated suspensions as above were

given to Dr. D. Ellis for *C. neoformans* examination in Adelaide. In addition a few slants from off-white, pink and 1 mm diameter light brown colonies were sent to him.

3.3 Results

In total, 1130 samples collected over wet and dry seasons from the following sources were analyzed: *E. confertiflora* (flowers shown in Plate 3.1), white gum (*E. alba*), ghost gum (*E. papuana*), *E. tereticornis*, kamarere (*E. deglupta*), coconut flowers and coconuts (*Cocos nucifera*), raintree flowers (*Albisia saman*) (Plate 3.2), bougainvillias (*Bougainvillea spp*), banana plants and flowers (*Musa sapientum*), legumes (*Peltophorum pteocarpus*), rubber trees (*Willughbeia coriacea*), frangipani (*Plumaria spp*), mango trees (*Mangifera spp*), okari (*Terminalia okari*), breadfruit (*Artocarpus communis*), Guttarda speciosa (*Rubicaciaeae*), casurina (*Casuarinaceae*), sawdust from sawmills, pigeons (*Columbia sp.*), lorikeet (*Trichoglossus haematodus*) and cockatoo (*Cacatuiane*), cuscus (*Phalanger mimicus* and *P. sericeus*) and Goodfellow's Tree-kangaroos (*Dorcopsis atrata*). Locations (National Capital District, Central Province and elsewhere), sample types and sampling periods are shown in Tables 3.1-3.3.

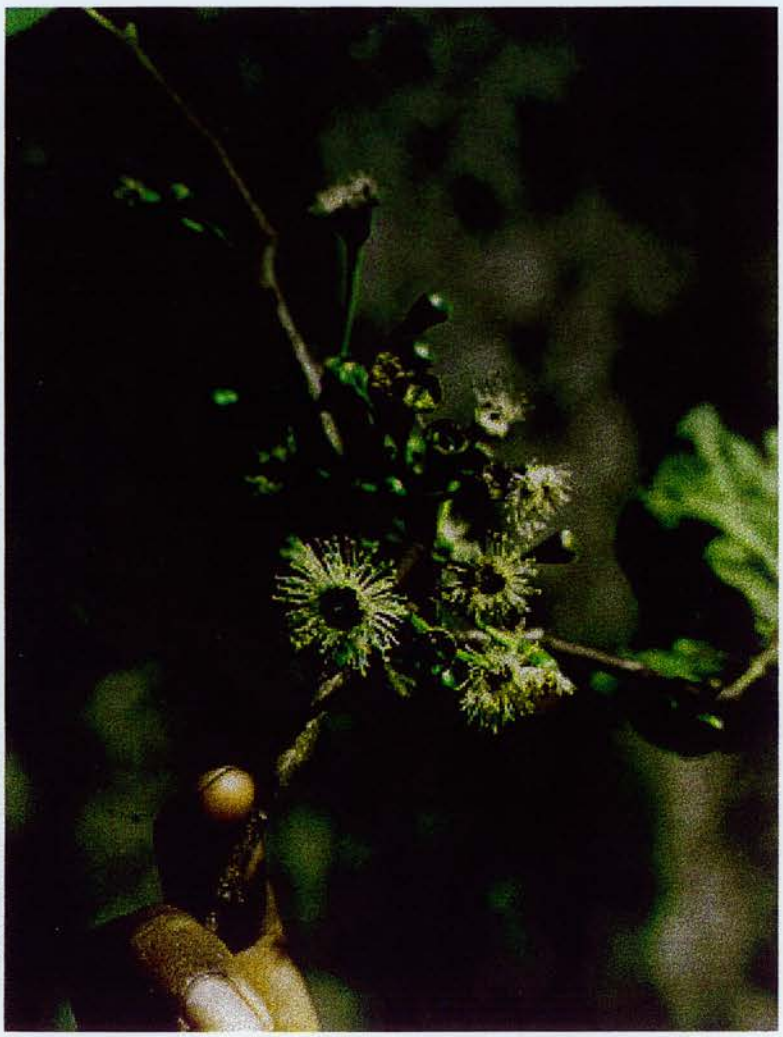

Plate 3.1 **Flowers of *Eucalyptus confertiflora*. One patient sat beneath such flowers during school breaks at Gerehu High School. He is holding the flowers.**

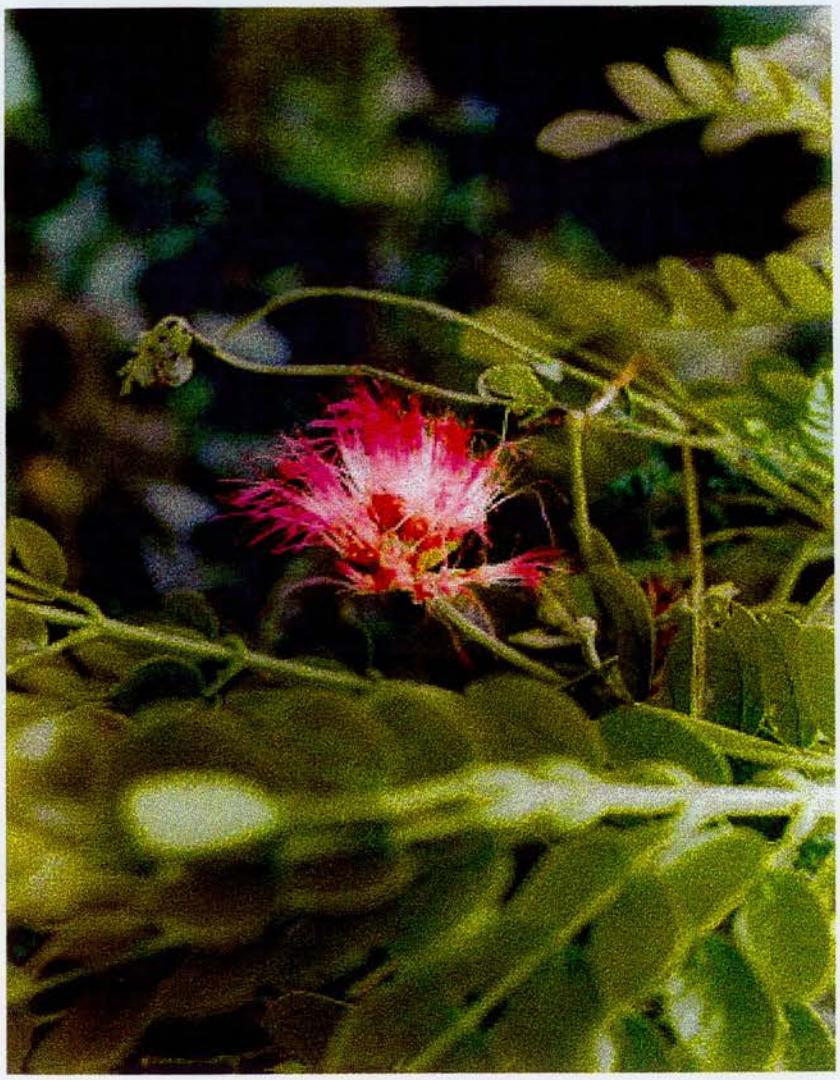

Plate 3.2 **Raintree flower *(Albisia saman)* in the grounds of PMGH**

Table 3.1 National Capital District specimens collected November 1991 - April 1994

Source/Specimen Type	Flowering[a]	Others[b]
E. alba	24	8
E. confertiflora	55	2
E. papuana	23	4
Eucalyptus sp.	108	370
Other plants & trees	53	41

Total 724 specimens, 594 Eucalypt associated. a: debris and flowers from trees/plants in flower. b debris from non flowering plants/trees. 67 and 11 repeated specimens from around homes of two patients. Further specimens not tabulated: patient's house dust/soil: 13, bird faeces (pigeons/cockatoo): 11 and 1 nest, marsupial faeces (cuscus/tree kangaroo): 11.

Table 3.2 Central Province specimens collected December 1991 - September 1993

Source/Specimen Type	Flowering[a]	Others[b]
E. alba	8	7
E. confertiflora	8	0
E. deglupta	0	6
E. papuana	5	3
E. tereticornis	24	43
Eucalyptus sp.	125	35
Other plants & trees	35	7

Total 336 specimens, 274 Eucalypt associated. a: debris and flowers from trees/plants in flower. b: debris from non flowering trees/plants. 47 (including some on repeated occasions) and 2 specimens from around homes of two patients. Further specimens not tabulated: patients' house soil: 5, sawdust: 6, bird faeces (pigeon/canary), marsupial faeces (cuscus/tree kangaroo): 2.

Table 3.3 Specimens taken elsewhere in Papua New Guinea May 1992 - April 1994

Province/Details	Sample type (quantity)	Source
Madang (CRI)	faeces (4)	Goodenough kangaroo, cockatoo, lorikeet
	flowers (1)	Guttarda speciosa
Morobe	tree debris (3)	*E. camaldulensis*
	tree debris (1)	*E. tereticornis*
Western*	faeces (47)	*P. sericeus Dorcopsis* sp, *P. forbesi*, unidentified marsupials
Rabaul	flower, plant debris, soil (14)	by patient's home

Total 70 specimens.
CRI: Christiansen Research Institute, Madang Province.* Frozen -20^{0}C.

Spiked control samples generally produced 10-30 typical BCE colonies per quarter plate. This is illustrated in Plate 3.3. These varied in size from 2-4 mm and became evident after 3-4 days culture, growing well in the presence of moulds. In some environmental samples there were off-white, light pink and brown yeast colonies grown, as well as moulds, some of which would stain the medium a light brown colour. None of these appeared similar to the BCE colonies of *C. neoformans* on control plates. With care the yeast colonies could be picked off. In none of these cases was *C. neoformans* identified. *C. laurentii* was the only *Cryptococcus* grown, particularly from *Eucalyptus* spp. All samples were negative for *C. neoformans*.

One case diagnosed with var. *gattii* infection was born in Wau/Bulolo in Morobe Province where there are a few *E. camaldulensis* surviving. One set of samples taken from these trees was negative. Further sampling was not possible for logistical reasons.

Plate 3.3 ***Guizotia abyssinica*** **creatinine (bird seed) agar exhibiting brown colour effect with** ***Cryptococcus neoformans*** **(round mucoid brown colonies) and lack of growth inhibition by other fungi.**

3.4 Discussion

Given the frequency of cryptococcal meningitis in PNG, it is surprising that environmental sampling using similar methodology to that used by Ellis and Pfeiffer has not yielded positive results for either variety. These authors shook environmental samples in 20mls sterile water, allowed them to stand for 10-15 minutes and streaked 0.5ml aliquots onto GACA, incubating at 26^0C for 7 days (Ellis and Pfeiffer, 1990b). They made 2,100 collections before finding *C. neoformans* var. *gattii*, but once alerted to an environmental source, were more frequently successful. Discussion with D. Ellis and examination of literature suggested that positive environmental samples yielded heavy and even semiconfluent growth of typical colonies though scanty smaller pinhead size colonies could occasionally be identified as *C. neoformans* (Dr.D.Ellis, personal communication). This investigation focussed on perceived likely sources, and hoped for more rapid success. Freezing for transport and storage of the 47 marsupial related specimens from Western Province and the use of smaller aliquots than Ellis and Pfeiffer could contribute to negative findings. Staib and colleagues have noted that some isolates of *C. neoformans* from AIDS patients and pigeon faeces fail to exhibit the BCE on bird seed agar (Staib et al. 1987). This may be due to the coexistent growth of other fungi leading to inhibition of the BCE by lowering the pH of the medium (Staib and Senska, 1973). All spiked control samples, exhibited the BCE on GACA without evidence of growth inhibition by other fungi. The investigation of non-BCE yeast colonies (no colonies showed typical morphology) revealed no isolates of *C. neoformans*. It is therefore unlikely that *C. neoformans* was missed because of growth inhibition by other fungi or misidentification of yeasts. The examination of flowering eucalypt associated suspensions by Dr Ellis with negative findings adds weight to these conclusions.

It would appear that *E. camaldulensis* is not the prime source of *C. neoformans* var. *gattii* in PNG, as there are so few surviving trees, although it is possible that this eucalypt could account for an occasional case (Chapter 2; Currie et al. 1990). The close relationship of *E. tereticornis* and its endemicity to PNG made this a likely source on epidemiological grounds, reinforced by the recent findings of *C. neoformans* var. *gattii* in association with this species in Australia (Pfeiffer and Ellis, 1992). It was not possible to confirm these associations in PNG. Since the distribution of var. *gattii* infections in PNG does not mirror the distribution of imported or endemic eucalypts it seems likely that the organism has a more ubiquitious distribution in nature. Recently Chen et al have demonstrated that the random amplification polymorphic deoxyribonuclear acid (RAPD) profile from infected patients with *C. neoformans* var. *gattii* in Arnhemland, North Australia differs from that of var. *gattii* isolated from Australian *Eucalyptus camaldulensis* and *E. tereticornis* (Chen et al. 1997). These eucalypts do not occur in Arnhem land, an area with a high incidence cryptococcal meningitis caused by *C. neoformans* var. *gattii*. These findings also suggest the existence of an alternative environmental source, other than known host eucalypts.

These results suggest that in PNG the environmental infectious propagules may be transiently present. Alternatively they may exist in a form that is not readily detected. Others too report lack of success in isolating *C. neoformans* var. *gattii* elsewhere in the tropics (Fisher et al. 1993; Swinne et al. 1994), although there has been a positive finding on one occasion in Brazilian bat guano (Lazera et al. 1993). No bat associated samples were investigated in the study described here. The latter group have also just reported persistent isolation of var. *gattii* from a hollow a pottery tree (*Moquilea tomentosa*) in Brazil (Lazera et al., 1998). Identification of the source(s) of *C. neoformans* var *gattii* as well as var. *neoformans* in tropical areas

should continue and may allow the development of strategies to avoid infection, particularly important where disease occurs both in healthy fit individuals as well as in the increasing numbers infected with HIV.

CHAPTER 4

Clinical epidemiology

of *Cryptococcus neoformans* meningitis

in Papua New Guinea

4.1 Introduction

Despite the continuing interest in cryptococcosis within PNG, particularly in Port Moresby, there had been no attempt to define clearly its epidemiology. The largest previous study in PNG (reported in a conference abstract) showed that 129 cases of cryptococcal meningitis were diagnosed from 1978-87 (Temu et al. 1987). There were 75 males and 64 females with ages ranging from 1.5 to 50 years. Thirty four of these cases were aged less than 15 years. No data on season of presentation or geographical distribution of cases were reported. Prior to this Slobodniuk and Naraqi presented a series of 13 cases from Central Province, of whom 5 were born in the Rigo Subprovince (Slobodniuk and Naraqi, 1980). This study describes the epidemiology of cryptococcal meningitis in PNG.

4.2 Methods

4.2.1 Case finding

Retrospective and prospective data was gathered from the literature, patient notes and prospective study cases. In PNG one of the most important features in the patient's social history is the village and region of origin. This indicates the likely mother tongue, beliefs, social affiliations and loyalties. In most instances this is accurately recorded in patient notes. The 'address' given by a patient at admission often simply indicates the contact point of a wantok (relative) who lives or works in Port Moresby. The area within the city, as opposed to a house number and street where the patient is based may be all that is indicated. Sometimes the address was not recorded in the inpatient notes. Patients considered prospectively at PMGH were asked where they had lived over the 6 months prior to admission. Although the incubation period of cryptococcal meningitis is not known, it is possible that exposure to the *C. neoformans* during this period might lead to clinical disease.

Visits, telephone calls or letters to the main hospitals in Madang, Lae and East New Britain helped to confirm details on patients presenting to these hospitals. PMGH laboratory cerebrospinal fluid results and Central Public Health Laboratory (CPHL) of Port Moresby cryptococcal latex antigen results were reviewed as available to identify and then trace records of patients identified. The CPHL receives samples from throughout PNG for cryptococcal latex antigen testing.

4.2.2 Case definition

A case of cryptococcal meningitis was defined as a patient in whom there were symptoms and signs of meningitis confirmed with positive cryptococcal findings on CSF microscopy and/or culture and/or serum cryptococcal antigen test. Biotype and serotype of *C. neoformans* isolates were also noted, when such information was available.

For the purposes of this part of the study, concentrating on epidemiological aspects, I identified cases defined in time, person and place and then tried to identify potential trends and correlations which might indicate clues as to the source(s) of infection.

4.2.3 Patient age

Until recently, in many hospitals and other healthcare centres, the age of an adult patient has not been recorded, simply because many patients do not know their age. Therefore an age recording of 'adult' is not uncommon. Health care workers are now encouraged to record an estimated age, as it is often useful to have some indication, even if not totally precise. In the data recorded here, ages such as 'adult' are not included in analysis.

4.2.4 Census and rainfall

Data was obtained from the 1980 and 1990 National Censuses, to provide denominators (Anonymous, 1982; Anonymous, 1993a). The University of Papua New Guinea Physics Department and National Weather Centre were able to provided rainfall data for Port Moresby.

4.2.5 Long term mortality

A retrospective group of 48 patients' virtually full records, including details of possible contact points, were available. These patients were followed up to find the long term mortality rate associated with cryptococcal meningitis in PNG. Wherever possible patients were visited, relatives contacted by telephone, writing or 'bush telegraph'. Tessi, the hospital social worker and the National Blind Association were valuable sources of information. In analysis it was assumed that patients who had left hospital within two weeks of presentation and diagnosis had received incomplete therapy and were probably dead.

4.2.6 Statistics

Data was handled on EpiInfo. The Chi squared (χ^2) test as described by Snedcor and Irwin, with Yates' correction was used to compare observed and expected numbers (Snedecor and Irwin, 1933; Yates, 1934).

4.3 Results

4.3.1 Demographic characteristics of all cases and isolates

Data was gathered on 96 cases of cryptococcal meningitis. These 96 cases were not consecutive as some sets of notes/results on further cases could not be located. There were 55 males and 41 females (ratio 1.3:1; PNG population 1.1:1), presenting from 1972-1993. The difference in numbers of males and females is not

significantly different from that anticipated from the PNG population ratio (χ^2 = 0.55, DF=1, p>0.5). The mean age was 26.2 years (standard deviation 13.3) with ages ranging from 6-60 years old. A total of 20 isolates were biotyped, 15 as *Cryptococcus neoformans* var. *gattii* (all serotype B) and 5 as *Cryptococcus neoformans* var. *neoformans* (all serotype A). Sixty two (64.6%) of the cases are known to have died.

All but 4 cases identified were immunocompetent on clinical, haematological and biochemical grounds. Those cases studied since September 1991, and some prior to this date were tested for antibody to HIV 1. Two male cases in late 1991 were identified as HIV 1 positive, and *Cryptococcus neoformans* var. *neoformans*, serotype A was isolated from both. One female case who died with cryptococcal meningitis in the first half of 1991 had previously had a renal transplant and was on immunosuppressive therapy. Her isolate was not biotyped. A further male case with var. *gattii* meningitis was found to have diabetes mellitus.

4.3.2 Incidence of cryptococcal meningitis

Estimates of the incidence of cryptococcal meningitis in PNG can be made from Temu's report (Temu et al. 1987). They identified 129 cases in PNG in the 10 years 1978-87, 12.9 per year, which, based on the 1980 Population Census gives a rate of 4.3 cases/million/year (Anonymous, 1982).

Eleven patients with cryptococcal meningitis presented from September 1991 to August 1992 to PMGH. Seven isolates were *C. neoformans* var. *gattii* and four var. *neoformans*, two in HIV1 positive men. This suggests that about eleven cases of cryptococcal meningitis present per annum from those actually living in CP and the NCD. Using Census figures from the 1990 Census, this gives an incidence of 33

cases/million population/year (Anonymous 1993a). If HIV infected individuals are excluded the incidence of cryptococcal meningitis is 27 cases/million population/year, with 21 *Cryptococcus neoformans* var. *gattii* infections/million population/year.

4.3.3 Patients' geographical distribution

4.3.3.1 Province and subprovince distribution

Many local patients spent some time in Port Moresby, and some time (e.g. at weekends) in their village, if it was within 4-5 hours drive of the city.

Table 4.1 Province of origin of 84 cases of cryptococcal meningitis and population distribution

Province	Population (% total)	Cases of CM (% total)
Chimbu	183,800 (5.1)	1 (1.2)
East Sepik	248,308 (6.9)	1 (1.2)
West Sepik	135,185 (3.8)	0
Enga	238,257 (6.6)	0
Southern Highlands	302,724 (8.4)	0
Western	108,705 (3)	2 (2.4)
Western Highlands	291,090 (8.1)	0
Gulf	68,080 (1.8)	14 (16.7)
Eastern Highlands	299,619 (8.3)	10 (11.9)
Manus	32,830 (0.1)	0
Madang	270,299 (7.5)	1 (1.2)
Central	140,584 (3.9)	36 (42.9)
Morobe	363,535 (10.1)	4 (4.8)
Northern	96,762 (2.7)	1 (1.2)
NCD	193,242 (5.4)	8 (9.5)
New Ireland	87,194 (2.4)	0
East New Britain	184,408 (5.1)	0
West New Britain	127,547 (3.5)	1 (1.2)
Milne Bay	157,288 (4.4)	5 (6)
North Solomons (WAR)	~145,000	0

Total Population of PNG = 3,529,538, Male:Females 1.1:1 (Anonymous, 1993a).
Patients with known Province of Origin =84

Table 4.1 shows the Province of Origin for all cases in PNG. Most cases originate from Central (36 cases, 42.9%), Gulf (14 cases, 16.7%) and Eastern Highland (10 cases, 11.9%) Provinces, and the NCD (8 cases, 9.5%). If the Chi squared test is applied, it is found that significantly more cases come from Gulf (χ^2 =9.4; DF=1; 0.01>p>0.001) and Central (χ^2 = 33.4; DF=1; p<0.001) Provinces, than would be expected. The number of cases originating from the NCD (χ^2 = 0.69; DF=1; p>0.1) and Eastern Highlands Province (χ^2 = 0.34; DF=1, p>0.5) are not statistically different from that expected, although Southern Highlands Province has significantly fewer cases (0 cases, χ^2 = 9.9; DF=1; 0.01<p<0.001) than expected.

Table 4.2 Population distribution and subprovince of origin of 32 cases of cryptococcal meningitis in Central Province

Subprovince	Population (% of CP total)	CM (% of CP total)
Abau	30,515 (21.7)	5 (15.6)
Goilala	21,812 (15.5)	5 (15.6)
Hiri	28,638 (20.4)	5 (15.6)
Kairuku	29,266 (20.8)	2 (6.3)
Rigo	30,353 (21.6)	18 (56.3)

CM = patients with cryptococcal meningitis. Total cases in Central Province (CP) 32. Table does not include data from the National Capital District.

Table 4.2 shows a similar breakdown for Subprovinces within Central Province. Plate 4.1 is a map of central Papua New Guinea, including Central Province. Within Central Province a significantly high number of cases was found in Rigo (18 cases, 56.3%. X^2 =6.99; DF=1, 0.01>p>0.001), and significantly low number of cases in Kairuku (2 cases, 6.3%. X^2 =8.89;DF=1; 0.01>p>0.001), whereas elsewhere the proportions were in keeping with the underlying population distribution shown in Table 4.2)

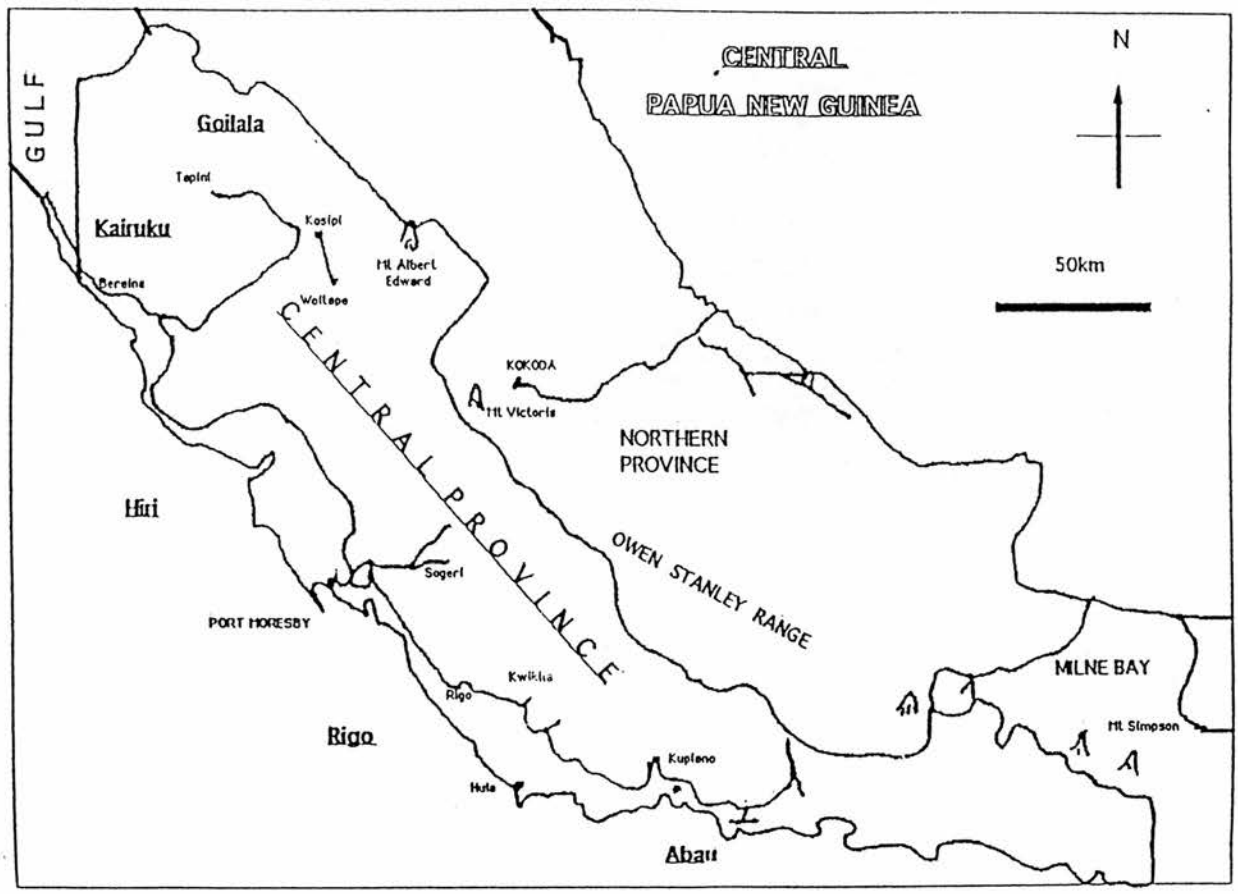

Plate 4.1 Map of central Papua New Guinea

4.3.3.2 Province of origin and biotype

The Province of Origin of 15 known var. *gattii* infected patients are considered for all of PNG. For seven patients the Province of Origin was Central Province, four originated from Eastern Highlands Province, and one each came from Morobe and Gulf Provinces, and the NCD. The five known var. *neoformans* meningitis patients originated from Central (2), Gulf (2) and Morobe (1) Provinces.

4.3.3.3 Recent residence and biotype

In addition, for some var. *gattii* patients the residence over the 6 month period prior to admission is known. These seven patients were resident in Central Province and the National Capital District. Four cases lived entirely in one location (2 in Port Moresby, 1 in Sogeri and 1 in Gerehu), whilst three cases spent time in both their home village (in Rigo, Abau and 9 Mile settlement), and in Port Moresby. In the latter three cases one cannot conclude where infection was acquired, even if it was thought to have been within the six month time period. Origin and recent address within Port Moresby showed no clustering. Three (including the two HIV 1 positive individuals) var. *neoformans* infected patients had been living in Port Moresby and two in remote villages within Central Province over the six months prior to admission.

4.3.4 Age structure of infected population

The age distribution of the population within each Province is not statistically different from that for the whole of PNG. This is compared with the age distribution of cryptococcal meningitis patients as shown in Table 4.3.

Table 4.3 Age distributions of PNG population and cryptococcal meningitis patients

AGE IN YEARS	<1	1-4	5-14	15-44	>44
POPULATION % (n = 3,529,538)	3.2	11.6	25	50	14.4
CM CASES % (n)	0	0	18.1 (16)	70.5 (62)	11.4 (10)

Mean age 26.2, SD 13.5 and range 6-60 years for the 88 cases. χ^2 is applied to numbers not percentages. χ^2 = 6.9; DF=1; 0.01>p>0.001 for difference in incidence in 15-44 year old age group, from that anticipated if cases were spread in proportion to population. χ^2 =1.17;DF=1; 0.5>p>0.1 if population aged 15 and over is considered separately.

No patients presented under the age of six years. Significantly ($p<0.01$) more patients were found in the 15-44 year age group than expected by comparison with the underlying population. Taking the population aged 15 and over years separately (i.e. all adult 'cases') , this finding still holds true, with $p<0.05$

4.3.5 Seasonal variation

4.3.5.1 All patients

Patient admission dates were well recorded, so the seasonal pattern of presentation can be described, as shown in Figure 4.1 for all cases that presented to PMGH. Patients presenting elsewhere in PNG are not included. An apparent peak occurs in May/June, with a second later in September/October. Neither of these peaks are significant if the Chi squared test is applied (for the June peak of Figure 4.1, X^2 = 0.38; DF=1; p>0.5). If var. *gattii* meningitis is associated with a specific seasonal environmental source then seasonality may be masked by confounding var. *neoformans* data. Removing the five known recent *Cryptococcus neoformans* var. *neoformans* cases from the data set showed a similar distribution to that in Figure 4.1.

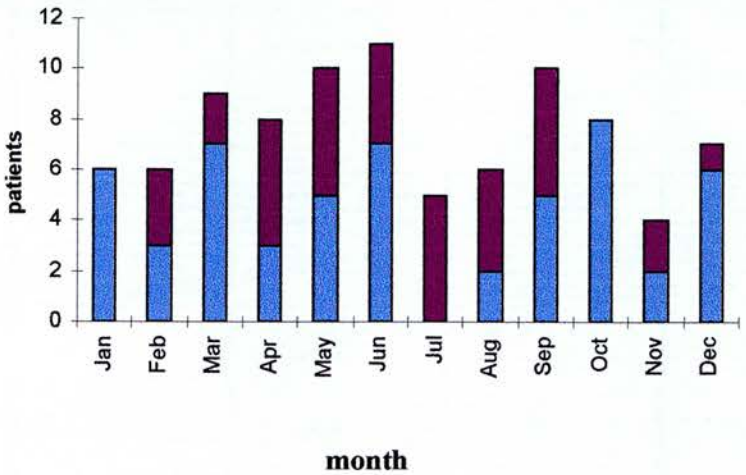

Figure 4.1. Month of presentation of 90 patients with cryptococcal meningitis at PMGH.
Years ranging from 1972-93. 36 females (■) and 54 males (■).

Breaking down the data into smaller groups reduces the numbers being analysed and only highly significant trends will be revealed. There is the possible benefit of uncovering significant correlations, which may otherwise be obscured by the mass of data. In an attempt to find such trends other subgroups were analysed.

4.3.5.2 Variety *gattii* infections

There were a total of 15 *Cryptococcus neoformans* var. *gattii* infected patients, 12 male and 3 female (ratio 4:1). Their mean age was 20.9 years (SD 12.6), with a range from 6 to 42 years old. Fourteen of these cases presented at PMGH. The month of presentation for these cases is shown on Figure 4.2. The seasonal distribution of cases suggests a 'peak' in March to May, with possibly another in September to October, consistent with the pattern shown in Figure 4.1. The numbers are too small to be conclusive.

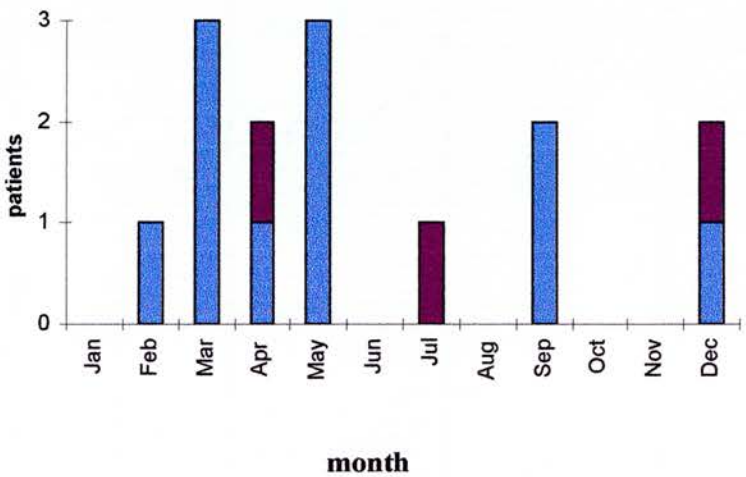

Figure 4.2 Presenting month with *C. neoformans* var. *gattii* meningitis. Mean age 20 years, SD 12.5, 3 females(■), 11 males(■).

4.3.5.3 Sex ratio and age grouping

The age and sex distribution in the under 15 year old age group is shown in Figure 4.3. There were no patients aged less than 6 years. The female : male ratio is 1.7:1, in reversal of the ratio of all the cryptococcal patients (F:M 1:1.1). In this group of patients there was seasonal variation in the presentation at PMGH. All female cases presented from December to March, and all males from April to September, with no overlap.

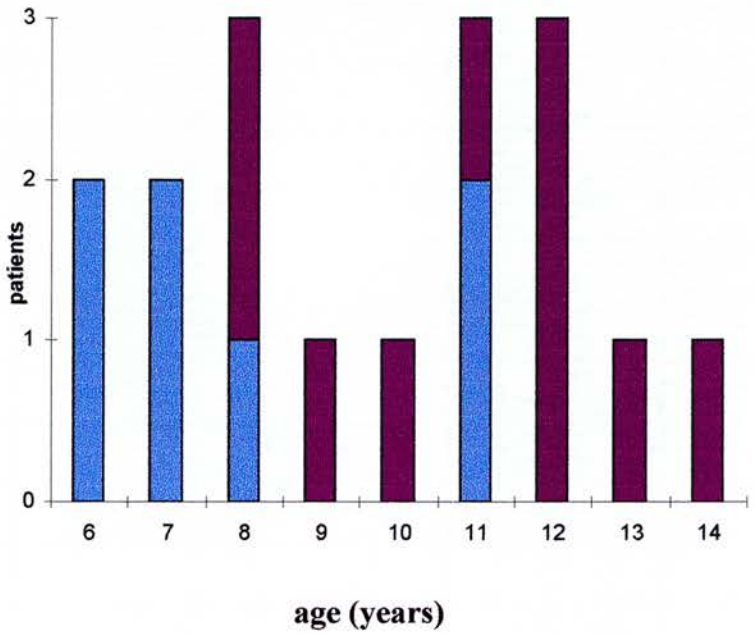

Figure 4.3 **Age and sex distribution of patients aged under 15 years. 17 cases, 10 female(■), 7 male(■). Mean age 9.7, SD 2.5. 6 isolates biotyped and serotyped; all were var. *gattii*, serotype B.**

This data suggests a possible difference in the age of presentation between the sexes, with males generally presenting aged 8 or under, and females from 8 years onwards.

Older age groups' seasonal, age and sex distributions reflected those of the total cryptococcal meningitis population. In the 15-30 age group 8 patients out of 40 presented in September, although this is not statistically significant ($p>0.05$).

4.3.6 Rainfall and month of presentation

Port Moresby and much of Central Province lies in the rain shadow of the mountainous Owen Stanley Ranges, and so precipitation is not high, with a mean annual rainfall of 1145mm annually between 1945 and 1985. This allows savannah to predominate along the southern part of the Papuan Region. Data from the Department of Physics UPNG as well as personal experience shows that there is considerable variation in rainfall, even within Port Moresby itself.

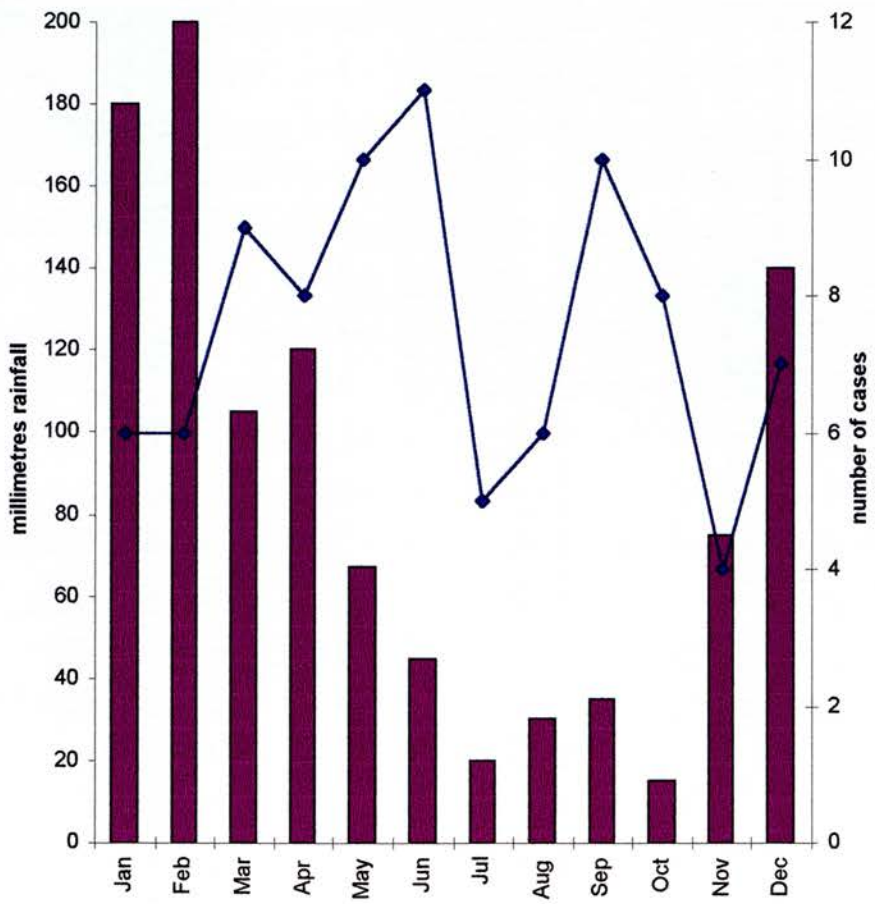

Figure 4.4 Month of presentation of all cases (◆) at PMGH compared with mean monthly rainfall (mm) (■) in Port Moresby 1945-85

As illustrated in Figure 4.4 the cases' presentation seems to peak in the dry season, rising from the end of the wet season, falling in July and August, which are dry, and then peaks again in September, before falling again as the rain arrives in November.

4.3.7 Long term follow-up

Follow up of past patients proved difficult, despite extensive efforts to locate them. Villages do not have regular formal communications with the outside world; street postal addresses are vague and difficult to trace. Travel, apart from natural hazards of flooding rivers, dirt (or no) roads, rough seas and dangerous flying, becomes increasingly awkward as one tries to avoid armed hold ups by 'rascals'.

The median age at presentation of the 48 adult cases presenting from 1979 to 1990, was 26 years, with a range from 12 - 60 years, male:female ratio 1.7:1 and duration of symptoms 1 day to 7 months. At follow up by August 1992, 15 (31.3%) were alive, 18 (37.5%) dead and 15 (31.3%) could not be traced. Considering the group in whom outcome was confirmed, five year survival of patients is shown in Table 4.4.

Table 4.4 Survival over 5 years in patients with known outcome

Years	0.5 (n=35)	1 (n=34)	2 (n=32)	5 (n=20)
Dead/Alive	15/20	15/19	15/17	11/9
% Alive	57	56	53	45

The causes of death were usually unknown and Standard Mortality Rate was not available for the general population.

4.4 Dicussion

4.4.1 Incidence

The figures derived from Temu et al.'s data suggests an incidence rate of cryptococcal meningitis for the whole of PNG to be 4.3 cases/million population/year (Temu et al. 1987). During the years 1978-87 the annual incidence appeared to rise (Temu et al. 1987). This was probably due to the increased clinical awareness and introduction of cryptococcal latex antigen testing. There had been no known cases of HIV infection. The figure of 4.3 cases/million population/year may well be an underestimate, as the diagnoses of cases were all confirmed at PMGH, CSF, serum and urine samples being sent from all over PNG. It is possible that some cases in remote regions and those without laboratories may remain undiagnosed, although in cases with gradual progression of symptoms and signs, there is time to reach more 'sophisticated' centres.

The rate of 4.3 cases/million population/year compares with rates of 2.5-5.0 cases/million/year in Victoria, Australia, 1.2 cases /million /year in Auckland, New Zealand, and 140 cases/million/year in the Aboriginal population of the Northern Territory of Australia (Speed, 1990; Speed and Dunt, 1995; Hutchinson et al. 1991; Fisher et al. 1993). The latter population is probably the most comparable to that in PNG, as there are similarities in both ethnic origins and environment. Amongst this Aboriginal population, the predominant variety of *Cryptococcus neoformans* is *gattii* (Fisher et al. 1993). Other series described from the tropics in the predominantly pre AIDS era from Singapore, Zimbabwe and Malaysia do not give overall comparable rates, but the rates deduced suggest that they are considerably lower than those in PNG (Tjia et al. 1985; Gould and Gould, 1985; Pathmanathan and Soo-Hoo Tuck Soon, 1982).

If it is thought that most of the cryptococcal meningitis seen at PMGH is acquired in or around Port Moresby, then the rate suggested by the current study is much higher at 33 cases/million population/year in Central Province and the National Capital District (27 cases excluding the recently diagnosed HIV1 patients), including 21 cases of *Cryptococcus neoformans* var. *gattii*. This level is most likely to be nearest to the actual rate during the study period, and is high when compared with most of the other rates mentioned above.

A subsequent 30 month study reported nationwide and Central Province incidences of 8 and 42.8 cases/million/year respectively (Seaton, 1996). This study included 4 HIV positive patients and may reflect the increasing incidence of HIV in PNG. Whether these high rates in PNG and Northern Australia are due to increased virulence of *C. neoformans* var. *gattii*, occult immunocompromise on the part of the local Melanesian population or high levels of exposure is not known.

4.4.2 Geographical clustering

The number of cases originating in Central (36) and Gulf (14) Provinces is significantly higher than those in Southern Highlands (0). The latter is lower than might be expected (Table 4.1). The Eastern part of Gulf Province is fairly accessible to Central Province and Port Moresby. The high number of cases originating from these areas could be explained by the fact that, despite movement of the population within PNG, by far the greatest proportion of people actually living in the NCD, Central and Gulf Provinces originate from these areas, and so they are more likely to present to PMGH. There are no Census or Hospital statistics available to confirm or refute this fact. Equally, they may also have had greater life time exposure if there is an environmental focus in these Provinces. They would be more likely to have acquired infection which then reactivated than individuals who have lived

some of their life elsewhere in PNG. In fact, one might expect more cases presenting from the NCD itself, if residence here conferred a greater risk of acquiring cryptococcal infection, but this is not the case. The increased incidence in those originating from Gulf and Central Provinces may reflect greater exposure or predisposition in these areas as well as access to PMGH.

There are no road links between the highlands and southern areas of PNG, including Central Province and the National Capital District. Eastern Highlands Province (EHP) has the best internal and external communications by road and air. There is a major hospital at Goroka, EHP, as well as road links to Lae and Madang hospitals. The low number of patients presenting from distant Provinces such as Southern Highlands Province could reflect logistical factors or those related to exposure.

Prior to this study, anecdotal suggestions have been made that a predominance of cases of cryptococcal meningitis seem to come from the Rigo Subprovince of Central Province. The numbers of patients with cryptococcal meningitis in the current study originating from Rigo (32) and Kairuku (2) subprovinces are significantly higher and lower respectively than expected (4.4.2.1.). Whether this is solely due to differing exposure to the source(s) of infection or not is not clear. A potentially confounding factor is the accessibility of these populations to PMGH, and the local availability of health care facilities to these populations. Northwest of Port Moresby, Hiri and Kairuku Subprovinces, which have a combined population of 57,900, (36.4% of CP population), together contribute only 7 (21.9%) cases, lower than expected when their population is taken into consideration. They have comparable air, road and healthcare facilities to those of Rigo, southwest of Port Moresby. The number of cases is disproportionately low when compared with Rigo, southwest of Port Moresby with comparable communication links. There is

therefore no evidence to suggest that the high number of cases originating from Rigo in particular can simply be attributed to ease of access to Port Moresby. A further way to ascertain the proportion of overall admissions originating from a particular region would be from hospital admission records, but such detail is not available.

The distribution of the *Cryptococcus neoformans* varieties *gattii* and *neoformans* infected patients by Province of Origin is consistent with the overall distribution of cases, as are the addresses of residence of these patients over the 6 months prior to admission. It should be noted that one *C. neoformans* var. *gattii* isolate came from a man originating from the village of Kuru who presented at Goroka Base Hospital Eastern Highlands Province. Other non-biotyped cases are known to have presented there, so it seem likely that there is a source in that vicinity. Trial plots of *E. camaldulensis* were planted near Goroka, and in the Whagi Valley (Chimbu Province), although they are reported to have fared poorly (Currie et al. 1990).

4.4.3 Age, sex distribution and exposure to *Cryptococcus neoformans*

The age distribution of cases may partly reflect the focus of attention on adult cases at PMGH. Although local clinicians are aware that paediatric cases occur in Madang, Lae and Port Moresby, they have not been previously described. The more detailed retrospective data available, including 13 cases from Slobodnuik and Naraqi's paper and the cases related to the 6 biotypes identified by Currie and colleagues in 1989, has focused on adult cases, and so the paediatric cohort may be under represented (Slobodniuk and Naraqi, 1980; Currie et al. 1990). This bias does not account for the possible excess of females in the under 15 year age group, nor the lack of documented cases aged less than 6 years. The latter is very much against the trend of infections in children which are often commonest in the very young. For example tuberculous meningitis is commoner than cryptococcal meningitis in

the PNG paediatric population, and certainly does occur in much younger patients (Professor J.Vince, personal communication). In a Malaysian series, the youngest patient with cryptococcal meningitis was 9 months old, but the biotype of the isolate not reported (Pathmanathan and Soo-Hoo Tuck Soon, 1982). The data presented here suggests that first exposure in PNG may occur at the age of 5 - 6 years, possibly related to some activity outside the home. There is no evidence for direct person to person spread of cryptococcosis (there are no close spacial or temporal clusters). The different ages of presentation in boys and girls under 15 (Figure 4.3.) suggests varying susceptibility or exposure, although the number of cases is too small to provide conclusive evidence.

The fact that all six isolates biotyped from the under 15 year old age group were *Cryptococcus neoformans* var. *gattii* is of interest suggesting that most infections in this group were with this variety. If this was the case, then their distribution and pattern may be of particular significance. It was not possible to identify any specific activity/exposure carried out by one sex but not the other at a particular age. Hormonal changes at puberty could be deleterious (males) or protective (females) as the apparent sex distribution of cryptococcal meningitis in the under 15 year age group (Figure 4.3.) reversed with female predominance, when compared with the overall study population. This is consistent with the earlier suggestion of the protective effect of oestrogens (De Wytt et al. 1982).

The study population's large over representation in the 15-44 cohort could be due to the adult bias in previous interest in cryptococcal meningitis in PNG. Considering the adult population only there is still a significant excess of cases occurring in the 15 - 44 year age group. However, this previous bias may not account for all of this predominance. A follow on study of 60 patients of all age

groups identified a cryptococcal meningitis incidence of 8 and 42.8 cases/million population/year in PNG and Central Province respectively with fewer cases in children under 12 years old and most in men (male:female sex ratio 2.2:1) (Seaton, 1996). Elsewhere in the tropics cryptococcal infection is reported to be commonest in the third decade of life, with male predominance, but infection rates are not compared with denominator populations (Lo, 1976; Gould and Gould, 1985; Tjia et al. 1985).

4.4.4 Seasonal variation of cryptococcal meningitis presentation and rainfall

The month of presentation of cases may reflect supposed (Eucalypt-associated) or other environmental exposure to *Cryptococcus neoformans* var. *gattii*, even though the incubation period is not known.

It was thought that by considering specific age groups, significant seasonal trends might be revealed. The main observation found was possible variation between the sexes in the season of presentation in children, females presenting from December to March, and males from April to September (4.3.5.3). No behavioural difference between sexes was identified that might explain such variation.

4.4.5 Season and eucalypt flowering

Initial discussions about Eucalypts raised the possibility of there being two flowering periods, one in the wet, and one during the dry season. Eucalypt flowering was documented in several locations. Within Port Moresby *Eucalyptus papuana* flowered in January, *E. confertiflora* in December to February and *E. alba* from March to June and again in November. Personal observations were not complete enough to definitely confirm biannual flowering, as a full year of informed observation was not quite possible. The dry season commences about April and

rainfall increases dramatically in November (Figure 4.4). Flowering seems to occur mainly during the wet season.

It is feasible that if there were a 2-3 month incubation period of cryptococcal meningitis following exposure, the seasonal variation documented here could be a reflection of release into the environment of *Cryptococcus neoformans* variety *gattii* associated with the flowering of one or more species of eucalypt, for example. This would support Ellis and Pfeiffer's hypothesis that this is an endophytic fungus associated with eucalypts (Ellis and Pfeiffer, 1990a). Further local longitudinal study of flowering and clinical correlation would be required to clarify any possible relationship between these two occurrences. The data on month of presentation with cryptococcal meningitis and rainfall does not suggest a clear link. Local microclimate varies dramatically within a few kilometers. Any potential effect of a particular tree's flowering on incidence of cryptococcal meningitis is difficult to discern.

4.4.6 Follow up and mortality

In those patients followed up the mortality rate of 43% in the first six months following diagnosis probably reflects deaths directly related to cryptococcal meningitis. The 55% 5 year mortality rate is difficult to assess without a suitable control group or even standard mortality rate for comparison. The high mortality rate may reflect later presentation than occurs in other healthcare settings or the pathogenicity of *Cryptococcus neoformans* var. *gattii.*

Overall these epidemiological studies confirm the magnitude of the problem of cryptococcal meningitis in PNG, and suggest clustering in one area and so a potential source of *C. neoformans* var. *gattii.* Possible seasonal peaks of presentation at PMGH were not associated with rainfall, while childhood infection patterns suggest that exposure or susceptibility to *C. neoformans* var. *gattii* varies with sex and age.

CHAPTER 5

Clinical studies of cryptococcal meningitis in Papua New Guinea

5.1 Introduction

Before the emergence of AIDS, cryptococcal meningitis in the tropics and sub tropics often occurred in apparently immunocompetent individuals, in contrast to those presenting in temperate climates, where infection was typically associated with immunosuppression (Lewis and Rabinovich, 1972; Lalloo et al. 1994). In both regions the meningitis was usually chronic and uniformly fatal if untreated. Survivors suffered a high morbidity often with permanent neurological damage. Biotyping of isolates of this yeast like fungus showed that *Cryptococcus neoformans* var. *gattii* (serotypes B and C) was commonly found in tropical areas, whereas *C. neoformans* var. *neoformans* (serotypes A,D and A/D) was found in temperate areas (Kwon-Chung and Bennett, 1984). In Papua New Guinea cryptococcosis was first diagnosed in 1946, since when it has been documented in animals and in apparently immunocompetent humans (Cox and Tolhurst, 1946; Champness and Clezy, 1962; Slobodniuk and Naraqi, 1980; Temu et al. 1987; Lalloo et al. 1994). Clinical isolates have been predominantly var. *gattii,* as elsewhere in the tropics and subtropics (Currie et al. 1990; Chapters 4 and 5).

This study reports the clinical findings and outcome in consecutive patients infected with *Cryptococcus neoformans.*

5.2 Methods

5.2.1 Patients and Setting

Port Moresby General Hospital (PMGH) is a 600 bed university teaching hospital, serving the local populations of the National Capital District (NCD - population 193,242, 1990 Census) and surrounding Central Province (CP - population 140,584) as well as acting as a referral centre for the rest of PNG (Population 3,529,538) (Anonymous, 1993a). All the patients described were Melanesians.

5.2.2 Diagnosis and assessment

From September 1991 to August 1992 confirmed cases of cryptococcal meningitis diagnosed by cerebrospinal (CSF) India ink staining (Plate 5.1), culture on Sabouraud's agar, and latex antigen testing (Crypto-LA Test, IBL), were studied prospectively. Patients had a full history and clinical examination. Whenever possible, accurate demographic data were obtained. Computed tomography (CT) and magnetic resonance imaging are not available in PNG. Regular assessment was carried out until discharge and thereafter at outpatient follow up. All isolates were biotyped using canavanine glycine bromothymol blue agar, then confirmed and serotyped by David Ellis and Tania Pfeiffer in Adelaide (Kwon-Chung et al. 1982b).

5.3 Results

5.3.1 Age, sex, HIV 1 status, *C.neoformans* variety and survival of patients

Isolates from all 11 patients diagnosed were biotyped. Details of age, sex, human immunodeficiency virus type 1 (HIV1) status, *C. neoformans* variety and survival are shown in Table 5.1. The four var. *neoformans* and seven var. *gattii* biotypes were serotyped as A and B respectively. One of the latter patients had previously undiagnosed diabetes mellitus. Two of the four var. *neoformans* isolates were from men who were HIV 1 positive, a third from a female with concurrent biopsy proven tuberculosis and the fourth from a male with *Plasmodium vivax* parasitaemia. No other concurrent conditions were found.

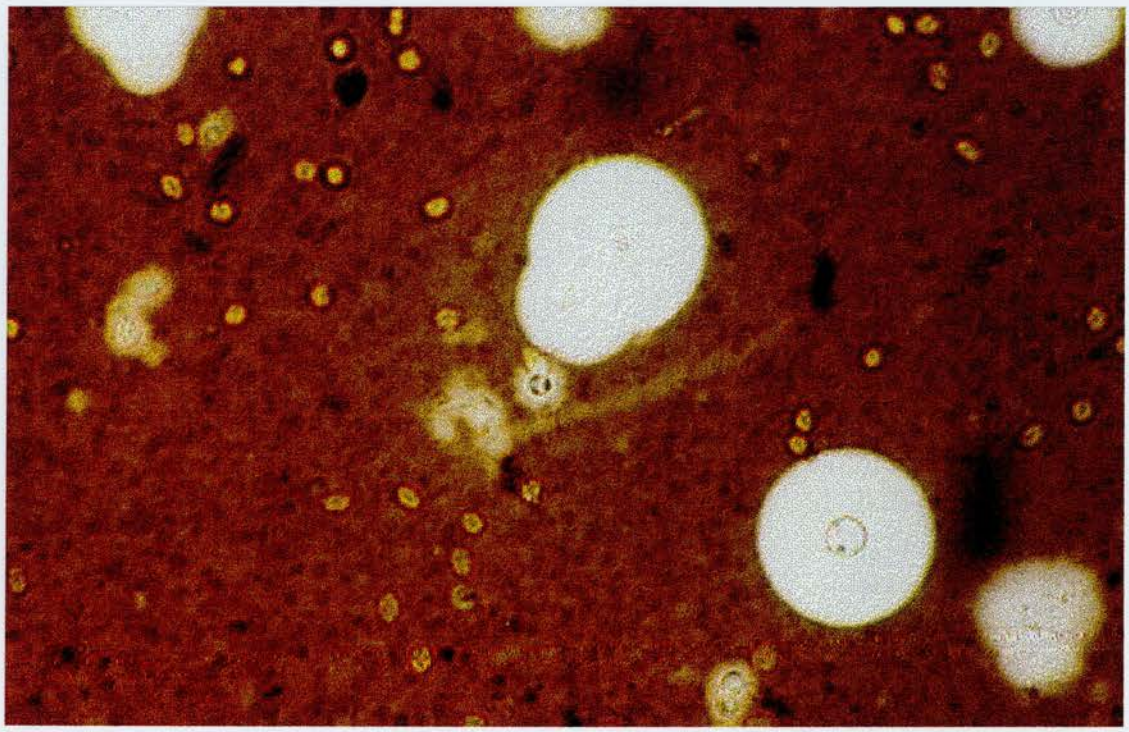

Plate 5.1 **India ink slide (x ~1000) of encapsulated *Cryptococcus neoformans* in the cerebrospinal fluid of Papua New Guinean patient. Polysaccharide capsule shows as clear white halo around yeast cells.**

Table 5.1 Age, sex, HIV1 status, *Cryptococcus neoformans* variety and survival

Patient No.	Age (years)	Sex	HIV 1 status	Variety	Patient's Survival*
1	18	Male	negative	*gattii*	yes
2	22	Male	positive	*neoformans*	no
3	21	Male	negative	*neoformans*	yes
4	35	Male	positive	*neoformans*	no
5	17	Female	negative	*gattii*	no
6	10.5	Male	negative	*gattii*	yes
7	35	Male	negative	*gattii*	no
8	18	Female	negative	*neoformans*	yes
9	40	Female	negative	*gattii*	yes
10	30	Male	negative	*gattii*	no
11	8	Male	negative	*gattii*	yes

Male:female ratio *gattii* = 5:2; *neoformans* = 3:1. Median age: *gattii* 24 years (range 8-40); *neoformans* 21.5 years (range18-35). *Completed treatment, discharged and have not represented by August 1995.

5.3.2 Initial Findings

Details of age, sex and *C. neoformans* variety with recent residence, birthplace and occupation are shown in Table 5.2. One patient with var. *gattii* meningitis presented and died at Goroka Base Hospital, Eastern Highlands Province; his CSF was examined at Port Moresby General Hospital, and the diagnosis made post mortem. The remaining 10 patients presented to PMGH and had been living within Port Moresby, NCD or the surrounding Central Province over the six months prior to presentation. The birthplace ranged from Eastern Highlands to Central, Gulf and Morobe Provinces. Occupation, exposure to birds, animals and plants and consumption of cigarettes, alcohol and betel nut were assessed, but no correlation with disease was identified.

Table 5.2 Age, sex, and *Cryptococcus neoformans* variety with recent residence, birthplace, and occupation.

Patient	Age (years)	Sex	Variety	Residence* past 6 months	Birthplace*	Occupation
1	18	Male	*gattii*	Gerehu, NCD	Manu Manu, Hiri, C P	Schoolboy Grade 10
2	22	Male	*neoformans*	Gordons, NCD	Lese Malalaua Gulf	Driver
3	21	Male	*neoformans*	Bogoromaka, Rigo, CP	Bogoromaka Rigo, CP	Villager
4	35	Male	*neoformans*	Port Moresby, NCD	Samazi Lae Morobe	Driver
5	17	Female	*gattii*	Kwapeupa, Rigo, CP & Boroko, NCD	Kwapeupa, Rigo, CP	Key Typist Student Grade 8
6	10.5	Male	*gattii*	Lalaura Abau, CP	Lalaura Abau, CP	Schoolboy Grade 2
7	35	Male	*gattii*	unknown	Fore Lufa EHP	unknown
8	18	Female	*neoformans*	Upiluma, Rigo, CP	Bunegoro Rigo, CP	Villager
9	40	Female	*gattii*	Erima Settlement, NCD	Biaru Wau Morobe	Domestic servant
10	30	Male	*gattii*	9 mile, NCD, & Kupiano, Rigo, CP	Moreguena Abau CP	Schooling attained Pre Grade 1
11	8	Male	*gattii*	Hohola, NCD	Lufa Goroka, EHP	Cook

NCD = National Capital District, EHP = Eastern Highlands Province, CP = Central Province, Gulf = Gulf Province. *Village, Sub Province and Province.

Clinical findings at presentation are shown in Table 5.3.

Table 5.3 Clinical findings at presentation

	var. *gattii*	var. *neoformans**	Both
Number of patients	7	4	11
Days of symptoms prior to presentation median (range)	37 (7-83)	7 (4-35)	28 (7-83)
Weight loss	3	2	5 (45%)
Fever	5	3	8 (73%)
Headache	7	4	11 (100%)
Vomiting	5	3	8 (73%)
Photophobia	2	2	4 (36%)
Neck stiffness	7	3	10 (91%)
Change in visual acuity	3	0	3 (27%)
Double vision	2	0	2 (18%)
Seizures	2	0	2 (18%)
Cough	0	1	1 (19%)
Pyrexia >37.5^0 C	2	4	6 (55%)
Altered mental status	0	2**	2 (18%)
Kernig's positive	4	2	6 (55%)
Papilloedema	3	3	6 (55%)
Cranial nerve lesion	2	0	2 (18%)
Long tract signs	2	0	2 (18%)

Data in brackets are percentages except where noted. *- one case minimal history available. **- Glasgow coma scores 10 and 11, who died and made a full recovery respectively.

Two patients had a past history of tuberculosis (not microbiologically confirmed) and seven were on antituberculosis treatment immediately prior to diagnosis of cryptococcal meningitis. Only one patient had concurrent tuberculosis, confirmed on neck lymph node biopsy. No patient had haemoptysis or dyspnoea at presentation. Systolic blood pressure was in no case over 130 mmHg. Cranial nerve lesions were found in two var. *gattii* patients at diagnosis (bilateral VIth, and XIIth palsies in one and unilateral VIth palsy in the other). Tunnel vision was reported in two patients and blurred vision in one other, but no objective abnormality in acuity could be demonstrated in those able to cooperate with assessment. Long tract signs were found in two fatal male var. *gattii* infected patients. No patient had an altered sense of smell at presentation.

In addition to the baseline data shown in Table 5.4, CSF pressure was measured to be > 30 cm CSF in one HIV 1 positive patient, and remarked to be "high" by the admitting resident in 6 others.

Table 5.4 Baseline CSF, cryptococcal antigen and culture characteristics

	gattii	*neoformans*	Both
CSF / no. measured			
Protein > 45 mg/dl	0/0	2/3	2/3
Glucose < 2.2 mmol	2/3	3/3	5/6
White cell count > 20/ul	3/4	2/3	5/7
Polymorph count/ul	6.5 (0-30)	11 (0-20)	9 (0-30)
Lymphocyte count/ul	40.5 ((0-240)	11 (0-188)	33(0-240)
pressure >20 cm CSF	0/1	1/1	1/2
Cryptococcal antigen			
Serum titre	6144 (128-16348)*	1536 (64-16,384	2048 (64-16384)
CSF titre	3042 (512-16384)*	512 (16-8192)	2048 (16-16,384)
"reactive"/ "<1024"	1/1	1/0	2/1
Extraneural site cryptococcal culture positive:			
Blood/ no. tested	0/4	0/0	0/4
Urine/ no. tested	0/3	2/3	2/6
Nasal/ no tested	0/2	0/2	0/4

Median values with ranges in brackets are shown unless otherwise indicated. CSF-cerebrospinal fluid. * - no data on 1 case.

Patient 6 had ventricular dilatation on cerebral ultrasonography at PMGH and CT scanning in Australia. In all patients the CSF India Ink stain and culture were positive. In one child (var. *gattii*) and one HIV 1 positive (var. *neoformans*) patient, no cellular CSF reaction was seen. CSF and serum cryptococcal latex antigen tests (Crypto-LA Test, IBL) were positive in all 10 patients tested. Urinary antigen tests were positive in 7 of 8 patients tested. In one HIV 1 patient and one with biopsy

proven tuberculosis, urinary culture was positive for *C. neoformans* var. *neoformans*. Neither CSF examination and culture nor blood culture was positive for other pathogens.

Four of six survivors had a Bacillus Calmette Guerin (BCG) vaccination scar. Mantoux testing with 10 units purified protein derivative (PPD) was negative in 5 cases, and 5 mm in the var. *neoformans* infected patient 3 with malaria at diagnosis and a past history of pulmonary tuberculosis. Patients 1 and 3 were subsequently re-tested during or after recovery, with induration of 11 and 20 mm respectively. Admission haematology and biochemistry results are summarised in Table 5.5. Blood films showed no evidence of haematological malignancy.

Table 5.5 Admission haematological and biochemical parameters

Parameter	range (median)
Serum sodium	129-138 (131) mmol/l
Serum potassium	2.6-4.3 (2.9) mmol/l
Serum urea	2.0-12.1 (4.0) mmol/l
Creatinine	0.06-0.16 (0.10) mmol/l
Haemoglobin	8.7-14.4 (12.4) g/dl
White blood cell count	8.7-15.5 (10.3) x 10^9l
Platelet count	140-504 (300) x 10^9l
ESR	54-65 (62) mm/hr

No clinical, biochemical or haematological evidence of malignancy or autoimmune disease were found. In 3 patients the CD4:CD8 lymphocyte subsets could be measured; only in one HIV 1 positive individual (4) was the ratio decreased; the other two adult male patients (one var. *gattii* and one var. *neoformans* with *P. vivax)* had normal ratios. Immunoglobulins were normal in 2 var. *gattii* cases measured, with a slightly raised IgM at 2.4g/l (normal range 0.5-2.0) and IgG at 21g/l (normal range 6-18) in the var. *neoformans* case with confirmed tuberculosis. Autoantibody screen was negative in 2/2 (1 var. *gattii* and 1 var. *neoformans*) cases. Chest radiographs were abnormal in 2 var. *gattii* and 1 var. *neoformans* cases, with apical lesion and right hilar reticular shadowing, left upper hilar opacity, and bilateral hilar infiltrates respectively. Examples of radiograph changes in one patient are illustrated in Plate 5.2.

No mycobacteria, cryptococci nor tumour cells were identified in sputum from these patients. Electrocardiographs were normal in 3 of 4 cases, with the fourth showing a PR interval of 0.18 seconds and left ventricular hypertrophy, which did not change with treatment.

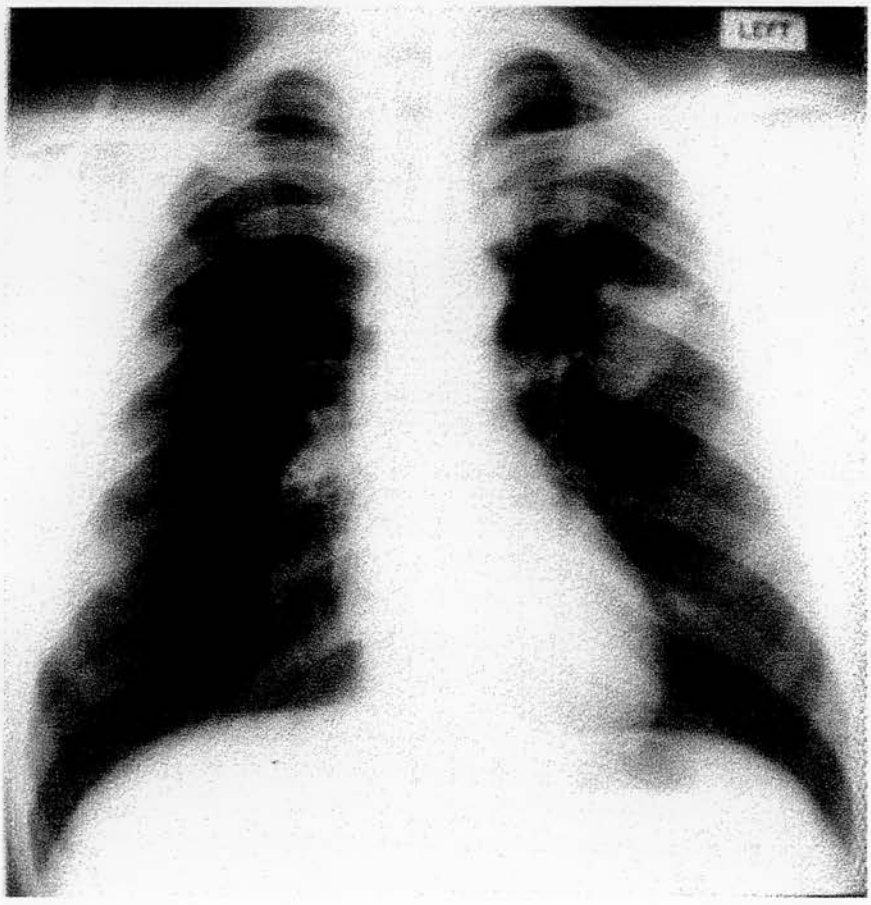

Plate 5.2 **Chest radiograph of a patient with cryptococcal meningitis showing left upper zone and right hilar infiltrate at presentation**

5.3.3 Treatment and outcome (Table 5.6)

Table 5.6 Treatment and outcome of patients with cryptococcal meningitis

	var. *gattii*	var. *neoformans*	Total
Male:Female	3:1	1:1	4:2
Amphotericin B* Total dose mg/kg	39.8 (27.6-69.1)	40.43 (30.3-50.6)	39.8 (27.6-69.1)
Steroid* at any time	3	3	6
Duration of treatment (d)	71 (52-121)	89 (74-104)	82 (52-121)
Cured/ no. cases (%)	4/7 (57%)	2/4 (50%)	6/11 (56%)
Death/ no. cases (%)	3/7 (43%)	2/4 (50%)	5/11 (45%)
Early < 1 day**	3	1	4 (36)
Late > 9 weeks**	0	1	1 (9)

Cured - had not represented by August 1995. Median values with ranges in brackets are shown. * Treatment received by the 8 patients who survived more than a day between diagnosis and death. ** - time between diagnosis and death. No case was treated without prior laboratory diagnosis

Survivors received between 0.3-1.0 mg/kg/day of amphotericin B parenterally, and 150 mg/kg/day flucytosine orally with the response monitored clinically and serologically. The two paediatric var. *gattii* cases were treated with a mean dose of 54.5 mg/kg of amphotericin B. Flucytosine levels could not be measured. Anorexia, nausea, vomiting, phlebitis, marrow suppression and azotaemia were particularly common. When possible the doses of antifungals were reduced in these cases. In six of the eight patients who received treatment, intravenous hydrocortisone was given immediately prior to amphotericin B in an attempt to reduce febrile reactions. Bilateral and unilateral sensorineural deafness was

detected during treatment in 2 var. *neoformans* and 1 var. *gattii* patients; this seemed to improve clinically after treatment. In 1 other var. *gattii* case bilateral deafness was noted clinically to correlate with the dose of amphotericin B.

Five patients (45%) died; four before or within 24 hours of starting amphotericin B and flucytosine, and a fifth patient who was HIV 1 positive, died 6 weeks after self discharge on oral ketoconazole, after he had received 57 days of combined flucytosine and amphotericin B treatment in hospital. Six (54.5%) patients, four with var. *gattii* and two with var. *neoformans,* survived to complete a full course of treatment. At discharge after completing treatment one patient with var. *gattii* was blind, with bilateral VIth and left upper XIIth motor neurone palsies, and was unable to respond appropriately to verbal commands, or make comprehensible sounds. He had dilated ventricles and probably had raised intracranial pressure at initial lumbar puncture. Five patients had no clinical deficit and had not represented by August 1995.

5.4 Discussion

Cryptococcal meningitis had not been previously reported in HIV 1 patients in PNG. In May 1991 a patient on immunosuppressive therapy following renal transplantation did present to Port Moresby General Hospital with cryptococcal meningitis. By December 1992 there had been 129 patients with HIV infection reported in PNG, but the patients reported here are the first found to be infected with *C. neoformans* (Anonymous, 1993b). Concurrent infection with HIV 1, tuberculosis or *P. vivax* may have predisposed these patients to opportunistic infection with *C. neoformans* var. *neoformans,* although coincidental infection is possible. One var. *gattii* patient had concurrent diabetes mellitus, but none had any other feature that might predispose to cryptococcal infections. The pattern in this series reflects that

already recognised of var. *gattii* infections occurring predominantly in apparently immunocompetent individuals in tropical areas. In central Africa in the pre AIDS era 12 of 13 isolates between 1951 and 1968 were var. *gattii*, whereas later 40 isolates biotyped from Zaire between 1970 and 1985 were all var. *neoformans* (Kwon-Chung and Bennett, 1984; 1955; Swinne et al. 1986b). It may be that the previous predominance of *C. neoformans* var. *gattii* and the rarity of var. *neoformans* infection in tropical areas was due to the absence of appropriately immunosuppressed individuals. Exposure to both varieties of *C. neoformans* may have occurred, but there were few hosts susceptible to infection with var. *neoformans*. In PNG the increasing exposure of individuals to conditions such as HIV infection and immunosuppressive treatment may lead to a rise in var. *neoformans* infections.

Prior to this study paediatric cases of cryptococcal meningitis have not been described in PNG. Both young patients here had var. *gattii* infection, and originated from similar environments within Central Province. The youngest biotyped (var. *gattii*) paediatric case of cryptococcal meningitis known in PNG is 6 years old, and the youngest case of cryptococcal meningitis reported in the literature is 9 months old, but the biotype is not described (Pathmanathan and Soo-Hoo Tuck Soon, 1982). Their occurrence confirms that childhood exposure occurs, although there is no evidence of vertical transmission.

In the tropics tuberculosis is often treated without microbiological confirmation. In seven cases, tuberculosis featured early in the differential diagnosis and treatment was started prior to the diagnosis of cryptococcal meningitis being made, although tuberculosis was subsequently confirmed in only one case. In PNG and the Northern Territory of Australia tuberculosis and lepromatous leprosy have been

reported concurrently with cryptococcal meningitis (Lo, 1976; Lalloo et al. 1994; Fisher et al. 1993). These experiences confirm the importance of positively excluding both tuberculous and cryptococcal infection in paediatric and adult cases presenting with subacute or chronic meningitis in a region where both are endemic. The diagnosis of one does not exclude the other. Where such cases are severely ill or do not respond to antituberculosis chemotherapy, cryptococcosis must be sought and treated without delay.

In the cases that had repeated Mantoux tests reactivity was markedly increased in convalescence. This suggests that cell mediated immunity in these cases improved with treatment of initial cryptococcal infection, and reduced the likelihood of undetected immunodeficiency.

Five cases had a lumbar puncture without ill effect despite papilloedema, allowing rapid diagnosis of cryptococcal meningitis. Earlier diagnosis and treatment may lower the mortality and morbidity. Where there is a high clinical suspicion of cryptococcal meningitis in countries without the facility or expertise for neuroradiological imaging and limited access to cryptococcal antigen testing the benefits of lumbar puncture may outweigh the risks, and indeed may be therapeutic.

Hearing loss and tinnitus are recognised as possible side effects of amphotericin B which may explain the sensorineural hearing reduction and deafness noted in our cases, although it might be expected to be bilateral in all such cases (Casscells, 1978; Stamm et al. 1987). Pre-existing or cryptococcal disease cannot be excluded as causes of this finding.

A subacute or chronic presentation was typical. Papilloedema occurred in 6 (55%) of cases. In none of the cases in this series was there clinical evidence of papillitis. Blindness ensued in only 1 (var. *gattii*) of 4 survivors with papilloedema at presentation. Raised CSF pressure was documented in 1 case, although the admitting resident commented that pressure was "high" in 6 others at lumbar puncture.

Recently in PNG blindness occurred in 9 (18.4%) of 49 patients (Lalloo et al. 1994). These 9 constituted 31% of patients who were fully treated. In the patient reported here blindness occurred prior to diagnosis, the rate of onset is not known and the CSF pressure was not recorded. It is uncertain to what extent direct optic nerve involvement, arachnoiditis or raised intracranial pressure resulting in nerve infarction may have been implicated. The latter aetiology may be significant in AIDS patients, as ultrasonography and CT scanning showed dilated ventricles, and slow visual loss seemed to be associated with intracranial hypertension (Rex et al. 1993). In such AIDS patients the rate of visual loss was 1.1%. In gradual onset blindness in these cases of cryptococcal meningitis aggressive treatment of rising intracranial pressure (ICP) such as shunting, nerve sheath fenestration, repeated lumbar puncture and acetazolamide therapy should be instituted when possible (Rex et al. 1993; Denning et al. 1991b; Garrity et al. 1993). Steroid therapy may also have a role in reducing ICP and inflammation (Rex et al. 1993; Denning et al. 1991b).

A retrospective study including those patients reported here has shown that in those with var. *gattii* meningitis patients treated at any point with corticosteroids visual deterioration and blindness occurred significantly less frequently, and in three patients vision improved as compared with those patients who did not receive

corticosteroids (Seaton et al. 1997a). In another study including these and other patients visual loss occurred in 52.6% of survivors and was associated with optic atrophy following optic disc swelling in 60.0%. Progression of disc swelling to optic atrophy was predicted by the presence of an abducens (VI cranial nerve) palsy and CSF cryptococcal antigen titres of >1:1024, but not with an opening CSF pressure of ≥300mm on admission (Seaton et al. 1997b). The contrasting rates of blindness in immunocompromised var. *neoformans* cases and immunocompetent var. *gattii* cases could be due to immune meditated optic nerve dysfunction in var. *gattii* meningitis caused by either compression due to arachnoid adhesion or oedema and inflammatory cell-mediated damage. Corticosteroids may have a role in preventing or halting visual loss in *C. neoformans* var. *gattii* meningitis in immunocompetent individuals.

The optimal antifungal regimen for var. *gattii* cases in particular is unclear. In PNG a lower dose of flucytosine (100 mg/kg/day) is now recommended to reduce toxicity. Ideally flucytosine levels should be monitored. Azoles and new amphotericin B preparations in particular are often not available. Even obtaining amphotericin B and flucytosine has become more difficult. Garlic has been used in the treatment of cryptococcal meningitis elsewhere in the tropics and China. Garlic and a Chinese commercial preparation, allitridium, containing mainly diallyl trisulphide, has been demonstrated in vitro to act synergistically with amphotericin B, demonstrating a possible clinical role especially where it is readily available (Shen et al. 1996).

The incidence of cryptococcal meningitis at PMGH is estimated from this study to be 21 var. *gattii* and 12 var. *neoformans* cases per million population/year from NCD and CP. A more recent estimate of predominantly var. *gattii* meningitis cases

in Central Province is higher at 42.8/million/year (Seaton, 1996). Further cases also regularly present at other centres throughout PNG. The incidence is lower than that of cryptococcosis reported in the aboriginal population of Arnhemland, Australia of 140/million/year, which contrasts with other reports of 28 in Victoria, Australia, and 1.2 in Auckland, New Zealand (Lo, 1976; Fisher et al. 1993; Speed, 1990; Hutchinson et al. 1991). A high rate of cryptococossis is also reported in Malaysia (Pathmanathan and Soo-Hoo Tuck Soon, 1982). The incidences may not be directly comparable, but exposure to and infection with var. *gattii* may be more frequent in tropical and subtropical areas. Whether infection is due to increased exposure, undetected immunocompromise or greater virulence is not clear.

The overall mortality rate of 45.5% in this series is similar to others from the tropics (Lalloo et al. 1994; Slobodniuk and Naraqi, 1980). Most deaths occurred soon after admission. In a series of 133 cases from Victoria in Australia none of 20 immunocompetent cases of var. *gattii* died and there was a higher incidence of cryptococcoma and non-fatal complications than in the var. *neoformans* cases (Speed and Dunt, 1995). When infection biotype is compared within the strata of host immunocompetence these distinctions become blurred (Speed, 1996). It may be that where cases present earlier to health care facilities many more var. *gattii* cases could be cured with fewer sequelae than is currently the case in many tropical centres.

Although this series of patients is too small to identify prognositic indicators, a larger series which has included these patients has now identified significant associations in *C. neoformans* var. *gattii* meningitis (Seaton et al. 1996). Mortality was higher in men and older patients, and associated with altered consciousness, convulsions prior to treatment and a maximum systolic blood pressure of >150

mmHg. This suggests that death may have been due to raised intracranial presure and subsequent tentorial herniation. Despite this, raised CSF opening pressure in 29/36 (81%) of patients did not predict outcome, nor did discharge serum cryptococcal antigen titres.

The source of *C. neoformans* infection remains uncertain in this series of cases. Var. *gattii* has been associated with *Eucalyptus camaldulensis* in Australia and California, and more recently with other related eucalypts. It has been suggested that the distribution of *E. camaldulensis* matched that of var. *gattii* meningitis, but this eucalypt is not indigenous to PNG (Chapter 2 above and (Ellis and Pfeiffer, 1990b; Pfeiffer and Ellis, 1991; Pfeiffer and Ellis, 1992; Heyligers, 1982)). All but one of this series lived in low lying savannah areas of Central Province. Three originated from near Goroka and Bulolo where there have been poorly growing plantations of *E. camaldulensis* and four from near sea level, where exposure to *E. tereticornis* may have occurred. Environmental samples around the patients' houses, from *E. papuana, E. confertiflora, E. alba* and *E. tereticornis* in NCD and CP, and from *E. camaldulensis* at Bulolo, as well as other plants, birds and animals have all been negative for *C. neoformans* (Chapter 3). Elsewhere *C. neoformans* var. *neoformans* has been most consistently associated with avian, particularly pigeon droppings (Emmons, 1955; Swinne-Desgain, 1975; Bauwens et al. 1986).

In conclusion, cryptococcal meningitis in PNG is commonly caused by *C. neoformans* var. *gattii* in immunocompetent children and adults. Malaria, tuberculosis and HIV 1 have been associated with *C. neoformans* var. *neoformans* infections, and should always be sought. Cryptococcosis and tuberculosis may present in a similar fashion and both must be considered. The source of cryptococcal infection in PNG has not been confirmed, but there may be some

geographical clustering of cases. Rapid presentation, diagnosis and treatment, with appropriate adjunctive measures, to lower raised ICP and prevent visual loss (possibly using corticosteroids) may help to reduce the high morbidity and mortality associated with this disease in the tropics and subtropics.

CHAPTER 6

Cryptococcus-human neutrophil interactions

6.1 Introduction

Following inhalation of the infectious propagule of *C. neoformans* into the lung, cellular defences are crucial in containing cryptococcosis. Neutrophils, monocytes and alveolar macophages play a major role in the host defence against *Cryptococcus neoformans* (Kozel et al. 1987; Levitz and Tabuni, 1991; Gadebusch, 1972; Miller and Mitchell, 1991; Collins and Bancroft, 1992).

Despite the presence of large amounts of C3 degradation products particularly iC3b, phagocytosis of encapsulated cryptococci by neutrophils and macrophages is limited. Richardson et al investigated differential binding of acapsulate and encapsulated strains of *Cryptococcus neoformans* to human neutrophils using a simple monolayer assay (Richardson et al. 1993). CR1 and CR3, the main receptors on neutrophils that bind C3b and iC3b respectively, have been shown to be associated with phagocyte cytoskeleton and to cluster (Detmers et al. 1987; Graham et al. 1989; Jack et al. 1986). C3 clustering occurred in a ligand-independant fashion and therefore to alter properties of the receptors it is possible that a second signal is required (Detmers et al. 1987). The host cell receptors and binding with *C. neoformans* are important in determining whether it will establish itself as a pathogen. The cytoskeleton influence in neutrophil fluidity and receptor clustering may be important in this interaction.

The clinical features of *C. neoformans* var. *gattii* and *neoformans* disease appear to differ. To explore these differences further in vitro studies of human neutrophil-cryptococcal binding were carried out, aiming to compare the neutrophil binding of

the two varieties of *C. neoformans*, developing techniques based on a monolayer assay (Richardson et al. 1993).

6.2 Methods

6.2.1 *Cryptococcus neoformans* isolates

Deep clinical isolates from Papua New Guinea, Vietnam and Edinburgh were studied. All have been biotyped and serotyped at Mycology Reference Laboratories (either Adelaide, Australia, or PHLS, England) and stored until use on Sabouraud's dextrose agar slopes or sterile distilled water at room temperature. Table 6.1 indicates details of isolates used in the initial set of experiments using the inverted cavity slide and Table 6.2 shows additional isolates used in Giemsa stained comparisons. In initial studies not all isolates had been serotyped and 2 var. *neoformans* were subsequently found to be serotype D (17D) or not typeable (22N/T). So that comparisons were also between known serotypes, these isolates were omitted from later assays.

Table 6.1 ***C. neoformans*** **isolates used in inverted coverslip measurements***

Isolate	NCPF	Biotype	Serotype	Site/Source/Country
16B	92.246	*gattii*	B	CSF/HIV-/PNG
17D		*neoformans*	D	CSF/HIV+/Edinburgh
19B	3798	*gattii*	B	CSF/HIV-/PNG
21B	3790	*gattii*	B	CSF/HIV-/PNG
22N/T		*neoformans*	N/T	CSF/ unknown/Vietnam
23A		*neoformans*	A	CSF/unknown/Vietnam
24A		*neoformans*	A	CSF/unknown/Vietnam
28B	3000	*gattii*	B	CSF/HIV-/PNG
29B	3756	*gattii*	B	CSF/HIV-/PNG
30A	3745	*neoformans*	A	CSF/HIV +/PNG

N/T - not typeable. * Some isolates used in subsequent Giemsa stained experiments

Table 6.2 Additional isolates used in subsequent Giemsa experiments

Isolate	NCPF	Biotype	Serotype	Site/Source/Country
14A	3745	*neoformans*	A	CSF/HIV+/PNG
18A	3799	*neoformans*	A	CSF/HIV-/PNG
26A	3748	*neoformans*	A	CSF/HIV+/PNG
31B	3746	*gattii*	B	CSF/HIV-/PNG

6.2.2 Preparation and standardisation of cryptococcal suspensions

Cryptococcus neoformans isolates were grown on 3% malt extract agar (Unipath) at 30^0C or 37^0C for 5-7 days in air or 5% CO_2. Morphology, in particular the degree of mucoid appearance of colonies was noted. Yeast suspensions were prepared in 0.85% (W:V) sterile saline and vortexed for 10 seconds to ensure a single cell suspension. This was then standardised to 1 x 10^5 yeasts/ml by haemocytometry and viable count and then diluted in Hanks Balanced Salt Solution (HBSS) (Sigma), pH 7.5. In each experiment var. *gattii* and var. *neoformans* strains were examined in parallel.

6.2.3 Capsule size measurements

Measurements were made on isolates 16B, 17D, 19B, 21B, 22N/T, 23A, 24A, 28B, 29B and 30A at both 30^0C and 37^0C. India ink was added to cryptococcal suspensions and measurements made using an eye piece micrometer of 40 cells per strain at x 100 magnification. Cell diameter (outer edge to outer edge of the cell wall) and the diameter of the cell plus the capsule (outer edge to outer edge of the capsule) were measured. The capsule thickness was calculated by subtracting the diameter of the cell from the diameter of the cell plus the capsule and dividing the difference by 2. The capsule volume was calculated by assuming that the shape was spherical and subtracting the cell volume from the volume of the cell plus the capsule.

6.2.4 Neutrophil monolayer preparation

Peripheral blood was obtained by venepuncture. Single drops of blood were placed on untreated, dust-free 16 mm coverslips (BDH) and incubated at 37^0C for 30 minutes in a humid atmosphere. Coverslips were then suspended in 37^0C 0.85% saline, blood clots removed and gently agitated, before rinsing in 0.85% saline to wash off remaining erythrocytes. This produced a 95% neutrophil monolayer on the coverslip, as established by Giemsa staining.

6.2.5 Serum and serum proteins

Peripheral blood collected from a volunteer was used as the source of normal human serum (NHS) containing opsonic complement components. This was prepared by placing approximately 40 mls of clotted blood in ice for 3-5 hours and then centrifuged at 2000 rpm for 15 minutes. The supernatant was removed, aliquoted into eppendorf tubes and frozen at -70^{0}C. Prior to use NHS was warmed to 37^{0}C. For experiments using absorbed serum with cryptococcal antibody removed, preparation was as follows. A plate with five day heavy growth of each variety was harvested and the resultant yeasts suspended in HBSS. The suspension was centrifuged at 2,000 rpm for 15 mins to pellet the yeast cells. Supernatant was removed, fresh centrifuged serum mixed with the pellet and left on ice for 1-2 hours. This removes any antibody present in the serum. The chilled mixture was then centrifuged as before and the supernatant aliquoted into eppendorf tubes and frozen in liquid nitrogen. Serum was heated at 56^{0}C for 30 min and re-equilibrated to 37^{0}C before use as the opsonin in those assays requiring heat inactivated NHS.

6.2.6 Binding assay and neutrophil binding

This was performed as previously described (Richardson et al. 1993). Briefly, neutrophil monolayers on coverslips were placed in wells of plastic tissue culture plates ("Repli", Sterilin) containing 1 ml of *Cryptococcus* suspension and 0-300 µl of opsonin. Most experiments used 100 µl opsonin. Plates were incubated at 37^{0}C for 30 min, monolayers washed in fresh warm saline to remove unbound yeast and then inverted onto a cavity slide. Ten random fields for each monolayer were observed under oil immersion phase contrast at x 100 objective. In the inverted

coverslip slide experiments assays were performed in triplicate, with one var. *gattii* and one var. *neoformans* isolate run in parallel in each assay. In binding assays where Giemsa staining was used, incubation periods varied from 0-120 minutes and normal human serum volumes from 0 - 300 µl as indicated. These assays were performed in duplicate or triplicate, again with up to two isolates of each variety run in parallel. At least 100 neutrophils in random fields were counted.

The total number of neutrophils, number of neutrophils with yeasts associated and total number of neutrophil associated yeasts were counted. It is difficult to distinguish whether the associations of neutrophils with cryptococcal cells are extra or intracellular (Richardson et al. 1993). Attempts to make this distinction and also deduce whether cryptococci were dead/alive and internal/external were made using acridine orange staining followed by quenching with crystal violet and examination by fluoresence microscopy (Goldner et al. 1983). This method has been used for this purpose in bacteria and subsequently in the yeast *Saccharomyces cerevisiae.* This did not differentiate cryptococci consistently and clearly enough and was not explored further. The yeast eukaryote DNA staining may be different from prokaryotes where this method has been more commonly used and so differentiation of internalised yeasts within neutrophils less clear than in studies of prokaryote internalisation. Therefore binding was measured rather than precise documentation of attachment or internalisation, since the latter could be very subjective. Two parameters were calculated:

1. The percentage neutrophils bound (%B) = (number of neutrophils with one or more yeasts associated)/ (total number of neutrophils) x 100. i.e. the percentage of the neutrophil population with yeasts attached.

2. The binding index (BI) = (total number of associated yeasts)/(number of neutrophils with one or more yeasts associated). e.g. if BI was 1, then binding was on a ratio of 1 yeast to 1 neutrophil.

6.2.7 Giemsa staining

The initial set of experiments counting yeasts and neutrophils were studied using the inverted coverslip slide method and the remainder of the binding experiments were carried out as follows. After the binding assay was completed and coverslips washed as before, they were allowed to air dry before Giemsa staining (10 minutes in methanol to fix cells, 3 minutes in 1:6 May-Grunwald stain (Sigma) followed by 3 minutes Giemsa stain (Sigma), washing in water between stains). Coverslips were then mounted on slides using DPX (diphenyl xylene, BDH, Poole) and the number of at least 100 neutrophils with associated cryptococci counted, usually in 3-4 fields at x 40 magnification. This method of staining and counting allowed handling of a greater number of coverslips per experiment and counting on different days. The duration of incubation and amount of opsonin were varied from 0-120 minutes and 0-300 μl respectively.

6.2.8 Statistical analyses

The significance of the mean of the results obtained was assessed by carrying out two tailed unpaired Student's *t* test on any results where two mean values were being compared. $P < 0.05$ was considered indicative of a statistically significant difference.

6.3 Results

6.3.1 Colonial morphology: mucoid appearance

Observation of colonies on plates showed that in general var. *gattii* isolates were more mucoid than var. *neoformans*, probably reflecting their greater capsular thickness. Ranking from most to least mucoid of 10 isolates grown for 5 days at 37^0C in air as shown in Table 6.3. This difference is illustrated in Plate 6.1.

Table 6.3 Ranking of mucoid appearance of 10 isolates

Isolate	**16B 19B=21B=12B**	**24A***	**28B***	**26A=18A=23A=14A**
Appearance	**mucoid >>**	**→**	**>>**	**non-mucoid**
variety	***gattii***	***(neof)***	***(gattii)***	***neoformans***

*24A *neoformans* and 28B gattii *exceptions* to the trend

When grown in air supplemented with 5% CO_2 the var. *gattii* colonies were more mucoid than when grown in air. Var. *neoformans* showed this effect to a lesser extent.

Plate 6.1 **Cultures of *Cryptococcus neoformans* var. *gattii* (16B) below and *neoformans* (14A) above showing difference in colonial mucosity**

6.3.2 Capsule measurements

6.3.2.1 Capsule thickness

Table 6.4 Mean capsule thickness μm (SD*) of 10 isolates in air at 30^0C & 37^0C

	Thickness (μm)		
	30^0C	**37^0C**	**p value**
var. *neoformans*	1.31 (0.5)	1.24 (1.36)	0.796
var. *gattii*	3.07 (1.36)	2.58 (0.81)	0.51
p value	0.042	0.018	-

*Standard deviation

Capsule thicknesses of 10 isolates: 16B, 17D, 19B, 21B, 22N/T, 23A, 24A, 28B, 29B and 30A were measured and compared. These data in Table 6.4 show that capsular thickness of var. *gattii* isolates is significantly greater than that of var. *neoformans* at both 37^0C and 30^0C. For each isolate the capsule thickness became smaller when grown in air with a rise in temperature from 30^0C and at 37^0 C. This difference was not statistically significant.

6.3.2.2 Capsular volumes

Capsular volume of the same 10 isolates was calculated. The volume of var. *gattii* was greater than var. *neoformans* as shown in Table 6.5, as expected from thickness and radius measurements. This difference did not reach statistical significance. It may been that if more isolates were compared, these capsular volume differences would become statistically significant.

Table 6.5 Mean capsular volume μm^3 (SD*) in air at 37^0C

	volume μm^3
var. *neoformans*	305.05 (112.1)
var. *gattii*	624.81 (431.6)
p value	0.17

*Standard deviation

6.3.3 Monolayer size and neutrophil content

The mean diameter of a coverslip monolayer was 1.05 cm with an average of 1254.5 neutrophils per monolayer. Neutrophils constituted approximately 95% of all cells remaining on the coverslips, as observed after staining with Giemsa.

6.3.4 Inverted coverslip assays

In all assays washing and counting was done after 30 minutes incubation of neutrophils with cryptococci. In the absence of NHS, or with heat inactivated serum, the binding of neutrophils to all isolates was very low (0.5-1%). The effect of NHS heat inactivation suggests that the opsonins involved in binding are complement.

The ten isolates 16B, 17D, 19B, 21B, 22N/T, 23A, 24B, 28B, 29B and 30A (five var. *gattii* and five var. *neoformans*) showed binding as in the Tables 6.6 and 6.7.

Table 6.6 Mean percentage neutrophil binding (SEM*) of isolates grown in air or with 5%CO_2 supplementation

	Percentage binding air	Percentage binding 5% CO_2	p value
var. *neoformans*	66.97 (5.9)	72.5 (5.09)	0.48
var. *gattii*	73.32 (4.7)	81.5 (2.95)	0.15
p value	0.41	0.14	-

* SEM = standard error of the mean

Although the percentage binding of var. *gattii* was greater than that of var. *neoformans* these differences were not statistically significant. Cryptococci grown in 5% CO_2 appeared to have a higher percentage binding, but again this difference was not statistically significant.

Table 6.7 Mean binding index (SEM*), of isolates grown in air or with 5% CO_2 supplementation

	Binding index -air	Binding index - 5% CO_2	p value
var.*neoformans*	1.215 (0.05)	1.05 (0.07)	0.07
var. *gattii*	0.76 (0.05)	0.70 (0.03)	0.29
p value	< 0.0001	0.0004	-

* SEM = standard error of the mean

The difference in binding index between varieties was significant when the varieties were grown in both air and 5% CO_2. Addition of CO_2 to the atmosphere in this way did cause a reduction in binding index for each variety, but this effect was not statistically signficant. In effect, although both varieties bound approximately the same proportion of neutrophils, greater numbers of *C. neoformans* var. *neoformans* than var. *gattii* cells were associated with neutrophils. Up to five neutrophils could be seen surrounding a single *C. neoformans* var. *gattii* cell, but fewer neutrophils were associated with each var. *neoformans*. This implies that after 30 minutes incubation var. *gattii* recruits more neutrophils around each cryptococcus than with var. *neoformans*.

6.3.5 Experiments using Giemsa staining

Although the inverted coverlip assay seemed reproducible, it necessitated rapid wet handling of coverslips. It was decided to try an alternative method of staining with Giemsa. This meant that once the coverslips had been washed following incubation, they could be dried and then fixed in methanol. Further handling and staining could be carried out over several days if necessary and so made the timetabling of assays and counting much easier with larger batches of coverslips. The remainder of the experiments were therefore carried out using this method of staining. Isolates used were grown in air. Plate 6.2 illustrates the appearance of Giemsa stained films, with cryptococci associating with neutrophils.

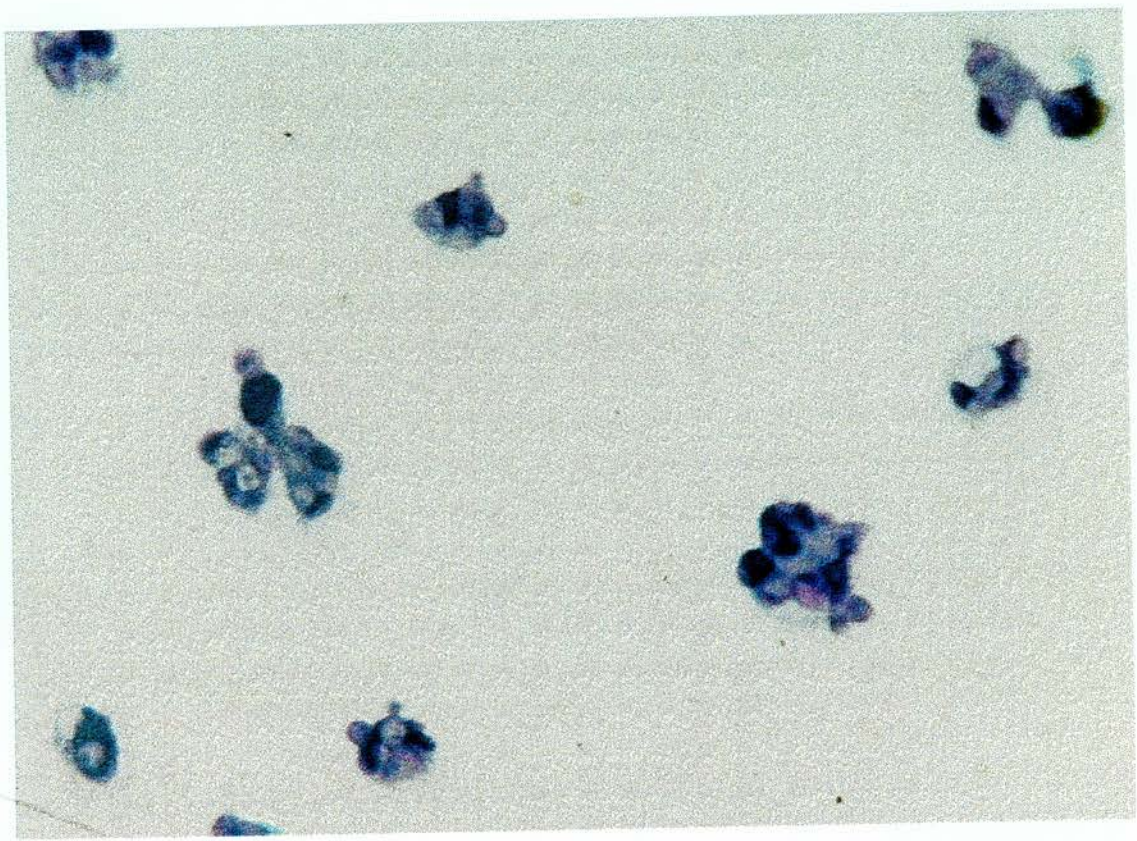

Plate 6.2 **Giemsa stain of cryptococcal-neutrophil interaction x ~ 2000 Dark neutrophils associated with lighter spherical encapsulated cryptococci**

Apart from the strain of *C. neoformans* used, there were many potential variables that could influence binding. Having established that the binding assay worked the conditions were varied to confirm that they were appropriate and optimal. As described in Section 6.3.6 paired var. *gattii/neoformans* isolates were used. The first variable considered was the effect of differing quantities of normal human serum used.

6.3.5.1 Normal human serum dose response (0-300 μl)

6.3.5.2 At 30 minutes

Two strains: one var. *neoformans*, serotype A (23A) and one var. *gattii*, serotype B (21B) were studied under the same conditions as previously, except for variation in the amount of NHS added to the mixture. Volumes of 0, 10, 30, 100 and 300 μl NHS were used. As shown in Figure 6.1 the percentage binding with no NHS added was 2% for both strains and percentage binding increased sharply to reach a maximum (45%, 47%) when 100 μl of opsonin was added. Increasing the quantity further to 300 μl did not increase the percentage binding of yeasts to neutrophils.

For both strains the binding index was 1 with no opsonin added, and reached a maximum at 30 μl, with no further increase at 100 μl or 300μl.

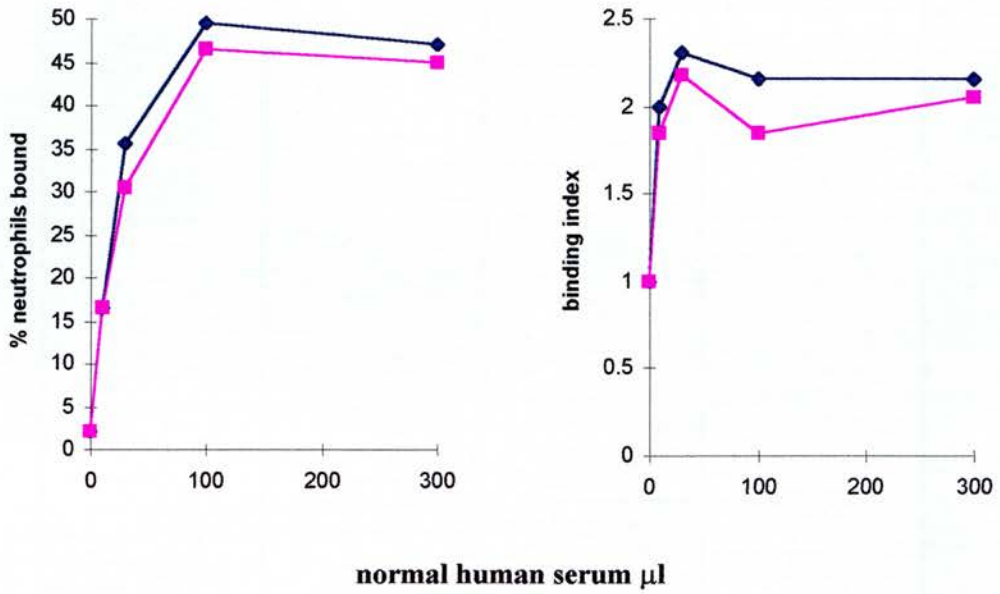

Figure 6.1 Binding of isolates 23A (◆) and 21B (■) at varying NHS volumes after 30 minutes incubation.

Maximal binding (percentage binding and binding index) at 30 minutes incubation of both varieties occurred at 100 µl. No increase was obtained by using 300 µl of serum. This suggested that at these concentrations of cryptococci and neutrophils maximal binding was occurring and that using more than 100 µl in subsequent assays would not alter binding.

6.3.5.3 At 120 minutes

Strains 14A, 16B, 23A and 29B were studied in 120 minute assays. Volumes of 0, 10, 30, 100 and 300 µl NHS were used. The results are illustrated in Figure 6.2. The percentage neutrophil binding increased to near maximal at 100 µl and approached the maximum (93-96%) when 300 µl NHS was added to the cryptococcal suspension. The binding index increased by 50% (i.e. 0.5) when increasing normal

human serum volume from 100 μl to 300 μl. Var. *gattii* appeared to have a lower binding index than var. *neoformans*.

These findings suggest that although the number of neutrophils bound rose little with an increase of NHS from 100 μl to 300 μl, more opsonisation of yeasts may occur at higher volumes of NHS allowing more cryptococci to bind each neutrophil in the longer, 120 minute assay.

The maximal binding found at 30 minutes does not seem to reflect maximal possible binding. This may be because of the time required for chemotactic signals to recruit further neutrophils to bind cryptococci. It would be anticipated that maximal cryptococcal-neutrophil binding is sterically limited and that the binding index would reach a maximal limit if the assay is allowed to continue over time.

As 100 μl NHS had been used in previous experiments and by Richardson et al and the difference in percentage binding between varieties appeared steady, this volume was used in subsequent assays (Richardson et al. 1993). In addition using larger volumes of NHS such as 300 μl would have become practically more difficult as much greater volumes of normal human serum would be required.

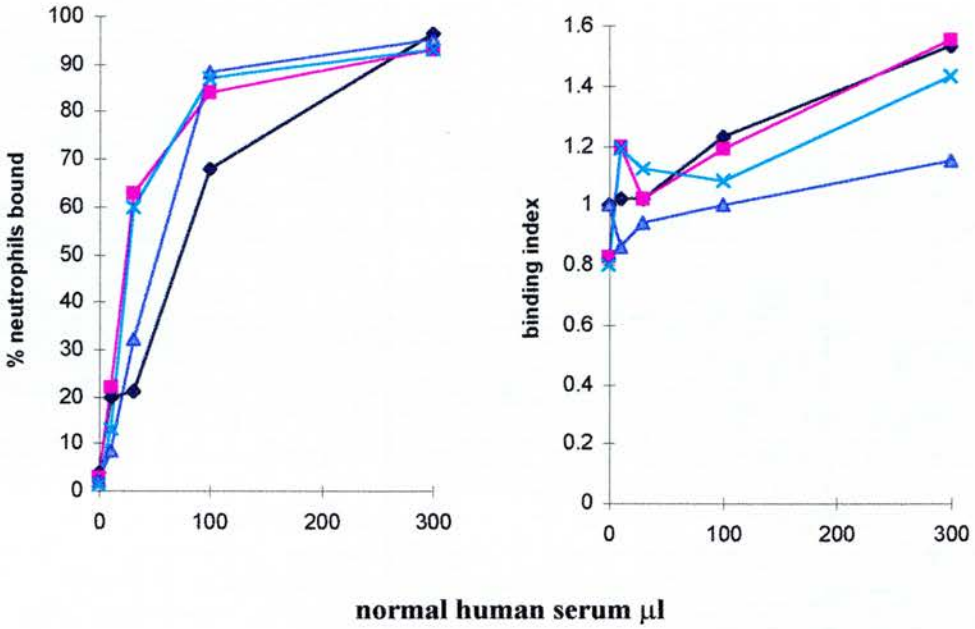

Figure 6.2 **Binding of isolates 14A (◆) 23A (■), 29B(▲) and 16B (×) at varying NHS volumes after 120 minutes incubation.**

6.3.5.4 Binding assessed at intervals with constant (100 µl) NHS volumes

In order to compare the rate of binding several time course assays were set up, assessing binding at intervals. An initial study over 90 minutes with two isolates was followed by further studies over 120 minutes.

6.3.5.5 Time course up to 90 minutes

Strains 23A and 21B, were studied at 5, 10, 30, 60 and 90 minutes incubation. Percentage neutrophil binding appeared to reach a maximum at 60 minutes, with a 2.5 - 3% decline at 90 minutes, whereas the binding index appeared to rise rapidly to 2 and plateau between 10 and 60 minutes. It then rose to 2.7 (isolate 21B) and 2.84 (isolate 23A) at 90 minutes, as shown in Figure 6.3.

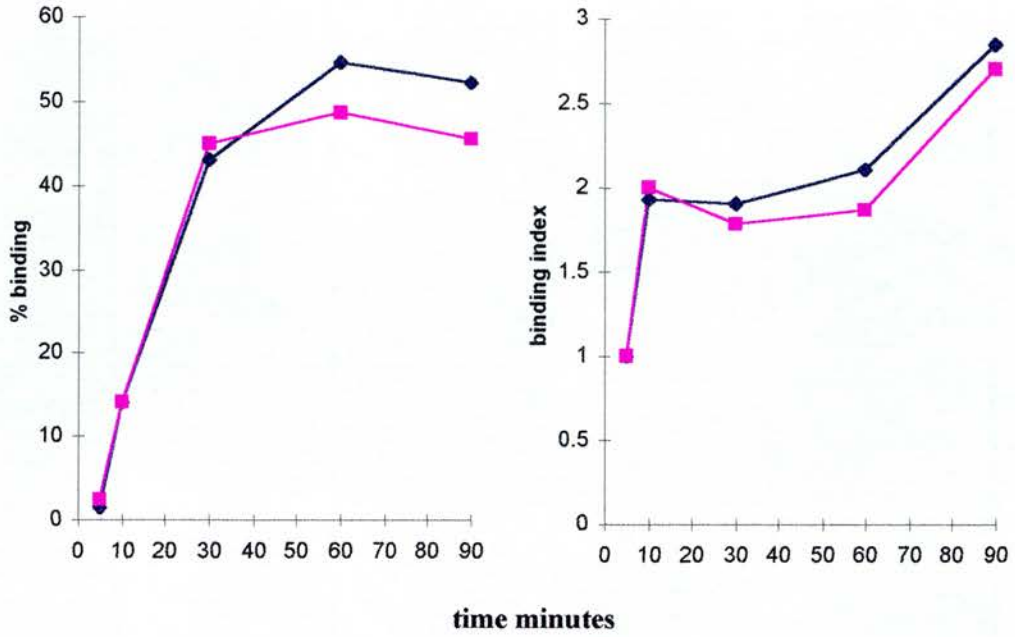

Figure 6.3 Binding of isolates 23A (◆) and 21B (■) over 90 minutes.

6.3.5.6 Binding of four isolates up to 120 minutes

Further timed assays were carried out with assessment at 0, 15, 30, 60 and 120 minutes using strains 22N/T, 26A, 28B and 31B, which comprise two var. *neoformans* and two var. *gattii* strains. As demonstrated in Figure 6.4 the percentage of neutrophils bound by cryptococci increased to a maximum at 60 minutes and this was maintained at the same level at 120 minutes. The var. *gattii* strains' maximum binding appeared to be 13% lower than the var. *neoformans* strains. This difference was consistent with, and the time to maximal binding (60 minutes) appeared similar, to that shown in the 90 minute assay.

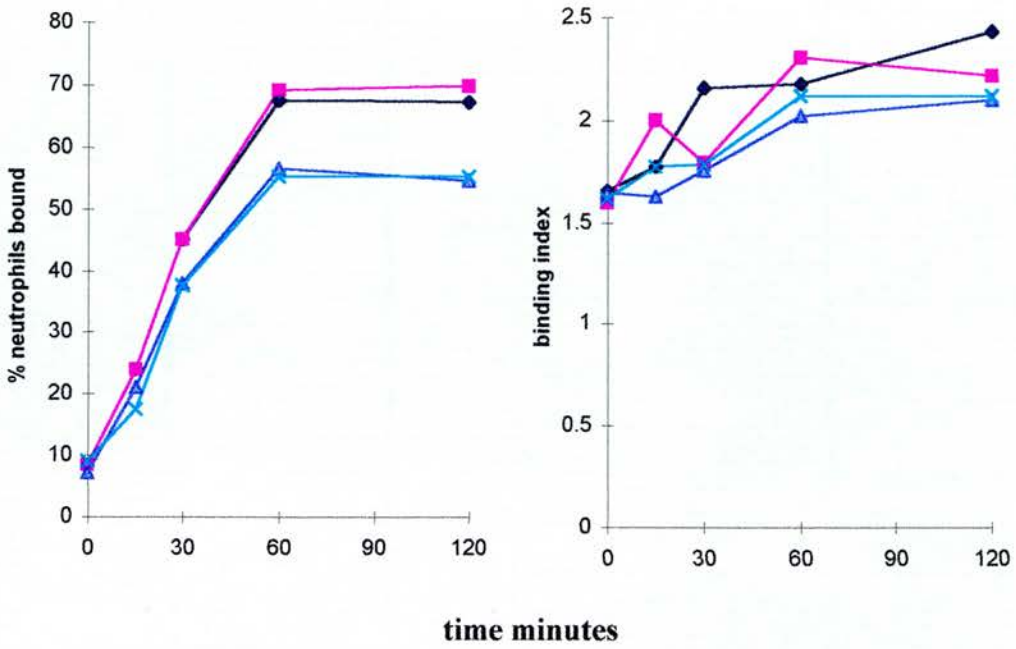

Figure 6.4 **Binding of isolates 22N/T(◆), 26A(■), 28B(▲) and 31B(×) over 120 minutes**

The binding indices are also shown in Figure 6.4. In this experiment they increased slightly to a maximum at 60 minutes that had changed little by 120 minutes. Although the 90 minute time point was not assessed in the 120 minute assay the marked rise in BI seen at 90 minutes in Figure 6.3 did not seem to occur by 120 minutes in this experiment. Overall the binding index of the var. *gattii* strains was marginally, but not significantly lower than that of the *neoformans* strains. Over the first 30 minutes there had been little difference between isolates, so subsequent assays omitted assessments at these times and instead included assessment at 90 minutes.

6.3.5.7 Binding of 8 isolates up to 120 minutes using absorbed NHS.

Two further experiments were run for additional isolates only of serotypes A and B, studying coverslips at 30, 60, 90 and 120 minutes for a further 8 strains, 4 each var. *gattii* and var. *neoformans*. The neutrophil donor in each experiment differed, but NHS source remained the same. Each experiment was carried out using absorbed NHS, as other experiments (data not shown) suggested that there are factors (probably antibody) present in normal human serum that also influence binding. Pre assay absorption of NHS with cultured cryptococci leaves no anti-cryptococcal antibody, so the only opsonin remaining is complement. This may account for the remaining low level binding seen after incubation with heat inactivated serum. The degree of influence may not affect each variety of *C. neoformans* equally.

Results shown are for at least one coverslip at each point. In these assays the maximum percentage of neutrophils binding yeasts as shown in Figures 6.5 and 6.6 is reached by 90 minutes and for three of four var. *neoformans* strains, 14A, 18A and 26A, was then reduced by 120 minutes incubation. The percentage binding of the remaining five isolates continued to rise to 120 minutes incubation.

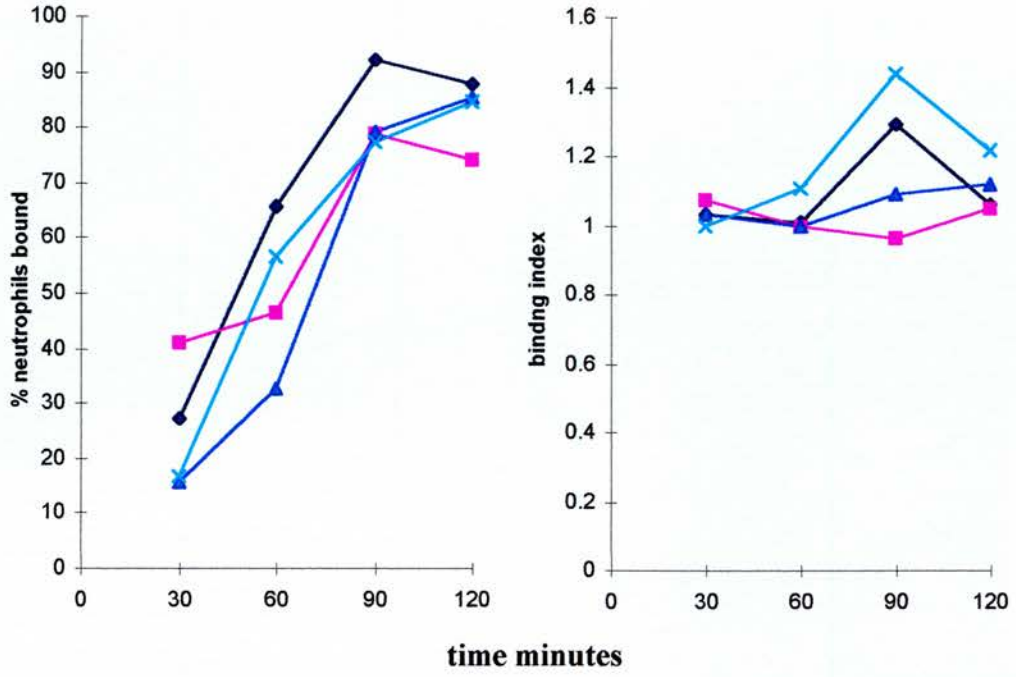

Figure 6.5 **Binding of isolates 16B (×), 18A (■), 26A (◆), 28B (▲) over 120 minutes**

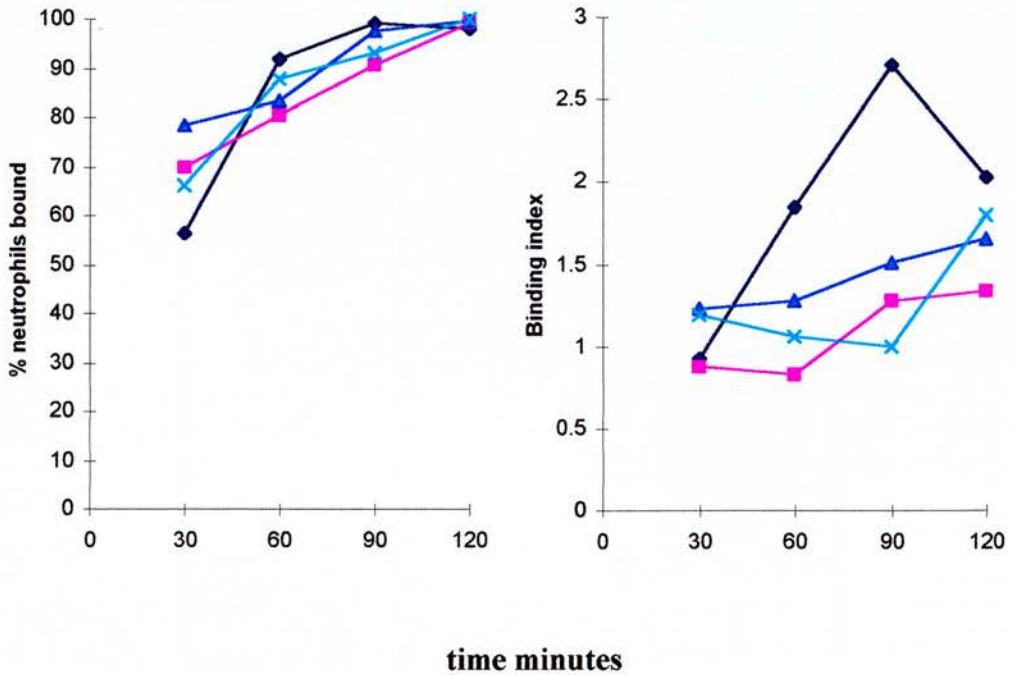

Figure 6.6 **Binding of isolates 14A (◆), 19B (▲), 23A (■) and 29B (×) over 120 minutes**

The binding index, as shown in Figures 6.5 and 6.6, rises with time up to 90 minutes incubation for all but two strains, 18A and 29B which decrease slightly. Three strains, 14A, 16B and 26A, then showed a reduction in binding index by 120 minutes incubation. There is considerable variation between strains with no clear pattern emerging.

There was no correlation between serotype/variety and binding index or percentage of neutrophils bound in these assays.

The rise in binding index at 90 minutes seen in the intial 90 minute assay, shown in Figure 6.3 was also found with some strains in these assays, shown in Figures 6.5 and 6.6. Isolate 23A consistently showed this rise in binding index in both the 90 and 120 minute assays.

6.3.5.8 Summary of time course experiments

Having found that the percentage binding was maximal at 90 minutes, yet binding index appeared to be still rising (Figure 6.3), further time course experiments with 100 µl absorbed normal human serum were carried out over 120 minutes, comparing varietal pairs of strains (Figure 6.4). These showed that the percentage binding and binding index maximized by 60 minutes and suggested that in these paired assays percentage binding and binding index of var. *gattii* was less than that of var. *neoformans*. In order to ascertain whether these findings were consistent and to increase the range of strains tested, similar experiments were set up to compare 4 serotype A and 4 serotype B strains, run in two separate assay runs (Figs. 6.5 and

6.6). Although these assays showed near maximal or maximal percentage binding and binding index at 90 minutes no clear difference between varieties was discernable.

6.4 Discussion

6.4.1 General comparison

The aim of the human neutrophil-cryptococcal experiments was to compare the interactions of the two varieties of *C. neoformans*. For all strains studied the presence of NHS was necessary for significant levels of binding to occur. If the NHS was heated to 56^0C binding was reduced to low levels, therefore complement is the main opsonin involved. Presence of opsonising antibody could account for low level binding. Alternatively the low levels of apparent binding in the absence of NHS or presence of heat inactivated NHS could be explained if the washing process was inadequate to dislodge all cryptococci from the coverslip. Such cryptococci would then be included in counts of associated yeasts if apparently touching neutrophils. In practice all yeasts appeared to be associated with neutrophils, none appeared to be isolated on their own, as would be expected to occur if washing was inadequate. Therefore it seems most likely that all cryptococci were genuinely 'associated' with neutrophils. In assays with no added NHS low level association was observed. This could occur if some other ligand other than complement or antibody sufficed for binding or neutrophils were able to generate low levels of complement.

In the 30 minutes assay the percentage binding and binding index were maximal with 100 µl NHS (Figure 6.1), however in the 120 minutes incubation maximal binding was not achieved with any volume of NHS up to 300 µl (Figure 6.2). The percentage binding was maximal by 60-90 minutes of incubation in the timed Giemsa stained assays (Figures 6.3-6.6). In all assays where the binding index was measured over varying times it tended to increase over time (Figures 6.2-6.6). Overall the results suggest that there may be significant differences between the varieties of *C. neoformans* in their cryptococcal-human neutrophil interactions, but further experiments would be required to prove this as the data is not entirely consistent as it stands.

6.4.2 Colonial appearance and capsule size

The degree of colonial mucoid appearance was generally linked to the capsule size of the isolate. Var. *gattii* appeared more mucoid than var. *neoformans* isolates, in keeping with their larger capsule sizes (Table 6.3). Isolates grown at 37^0C had smaller capsules than at 30^0C (Table 6.4).

Within varieties, it is known that capsular sizes vary considerably, demonstrating considerable phenotypic variation. Few direct comparisons have been made of *C. neoformans* var. *gattii* with var. *neoformans* isolates grown under the same conditions, but the greater capsule sizes of *C. neoformans* var. *gattii* has been previously noted (Young and Kozel, 1993). In that study, these differences were not highlighted, although in 15 encapsulated strains examined (3 of serotype D and 4

each of serotypes A,B and C), ranking of capsule sizes showed that var. *gattii* isolates were usually bigger than var. *neoformans.* In the current studies a similar and statistically significant difference in mean capsular size was found. Such a difference may play a role in the differing pathogensis of the two varieties. However, despite the fact that human cases of cryptococcosis due to minimally encapsulated var. *neoformans* isolates have a stronger host response, greater inflammation and less severe disease (Farmer and Komorowski, 1973; Levinson et al. 1974; Milchgrub et al. 1990), cryptococcal virulence has not always been correlated with capsule size (Dykstra et al. 1977).

Kozel et al have recently illustrated the importance of opsonization conditions on C3 deposition and phagocyte binding of large and small-capsule *Cryptococcus neoformans* serotype A cells (Kozel et al. 1996). They noted that previous studies demonstrated that following opsonization with NHS, phagocytes bind greater numbers of small-capsule than large-capsule *C. neoformans* cells. A single strain of *C. neoformans* was grown under conditions to produce large or small capsules and incubated for 30 minutes at various concentration in NHS. At low concentrations of yeast cells (125 per μl of NHS), the deposition of C3 fragments per unit of capsule volume and the binding of yeast cells to human monocytes was similar for yeast cells with large and small capsules. At higher cell concentrations, large-capsule cells exhibited suboptimal coating with C3 fragments and markedly diminished monocyte binding compared with small-capsule cells. Therefore they suggest that the inverse correlation between capsule size and phagocyte binding can be overcome by conditions promoting optimal C3 deposition.

Another way of stimulating phagocytosis of encapsulated cryptococci is by using cytokines (Collins and Bancroft, 1992). These scientists found that stimulation of murine peritoneal macrophages with tumour necrosis factor alpha and granulocyte-macophage colony-stimulating factor promoted complement-dependent ingestion of encapsulated cryptococci. These cytokines act on the phagocytes to reduce the amount of serum required for effective opsonisation of encapsulated cryptococci. With limited numbers of cryptococci and sufficient production of cytokines opsonisation of encapsulated cryptococci and binding to phagocytes is effective, but the balance is easily disrupted by an excess of yeast cells. Serum complement depletion occurs in vivo (Macher et al. 1978), which may be an important factor potentiating cryptococcosis in patients with widely disseminated disease, such as patients with AIDS (Chuck and Sande, 1989).

6.4.3 Quantities of yeast cells and NHS

In the current studies the number of yeast cells/μl NHS varied from infinity when no NHS was added, to 333 yeast cells/μl when 300 μl NHS was used. The most frequently used ratio was 1000 yeast cells/μl as used by Richardson et al (Richardson et al. 1993). In the current studies mean capsular volumes ranged from 132-1268 μm^3. These volumes are comparable to the small volume capsules of Kozel et al's study (Kozel et al. 1996). Their data with a single *C. neoformans* strain suggests that on large volume capsules (3,400 +_ 1300 μm^3) C3 binding may be suboptimal, as compared with small volume capsules (1,200 +- 790 μm^3) when

more than 250 yeast cells/μl NHS are used (Kozel et al. 1996). At such low capsular volumes in the presence of 1000 yeast cells/ μl NHS binding of C3 fragments is not suboptimal at 30 minutes incubation. Therefore binding of neutrophils in the current studies at this time is unlikely to have been limited by the amount of NHS present. This is demonstrated in the 30 minute assays in this study.

6.4.4 Inverted coverslip assays

Initial 30 minute binding assays, counted by inverted coverslip, were carried out on isolates grown in air and with 5% CO_2 supplementation. The latter conditions mimic more closely the conditions in the lung and tissues. With heat inactivated NHS or no NHS, binding was undetectable. Therefore in this assay complement was required for binding to take place.

Slight, but not significant increases in binding index and percentage binding were noted with the increase in CO_2 When grown under either set of atmospheric conditions and counted using the inverted coverslip method there was a non significant difference in percentage binding (var. *gattii* was greater), and significant difference in binding index (var. *neoformans* being greater). Fewer neutrophils were associated with each var. *gattii* cell than with each var. *neoformans*. In these assays the cryptococcus:neutrophil ratio was 100:1, so the number of yeast cells should not limit their binding to neutrophils. In this assay opsonisation with normal human serum is not limiting either, so binding differences could reflect either limiting steric binding, differences in complement binding or recruitment of neutrophils. These three possibilities are considered below.

Richardson et al found that unlike acapsulate cryptococci, opsonised encapsulated serotype A cryptococci compete for binding to neutrophils. This requires actin, involves neutrophil membrane ruffling and does not appear to lead to phagocytosis of the yeast (Richardson et al. 1993). It was proposed that cryptococcal competition, probably involving complement receptors, may limit binding, rather than steric factors.

Young and Kozel have shown that the variations in capsular structure that characterise each serotype have no significant influence on the maximum amount of C3 that can bind by 80 minutes ($C3_{max}$), but that the rate of C3 deposition depends significantly on the serotype (Young and Kozel, 1993). C3 accumulates faster on cells of serotypes A and D than on cells of serotypes B and C. They found a significant but not linear correlation between capsular volume and $C3_{max}$. It could be that slower C3 binding on var. *gattii* serotypes in the 30 minute assay in this study lead to the cryptococcal-neutrophil binding index being lower. Laxalt and Kozel reported that incubation of encapsulated cryptococci in normal human serum lead to release of soluble cleavage fragments that are chemotactic for neutrophils (Laxalt and Kozel, 1979). This chemotactic factor was then shown to be blocked in *C. neoformans* -activated serum by treatment with antibodies specific for C5, but not by antibodies specific for C3 (Diamond and Erickson, 1982). This identified C5 as the essential chemotactic factor.

6.4.5 Giemsa assays

In the subsequent Giemsa studies, variable differences in binding between the two varieties were found. There are several possible explanations. It may be that early experiments showed significant differences in percentage binding and binding index between varieties by chance. By selecting a p value of <0.05 to denote significance will allow 1 in 20 similar comparisons to give a 'significantly different' result. A significant difference could by chance be obscured, or the isolates used in expriments may not be representative of the variety in general. It may be that as experiments were carried out over a considerable period, inter and intra person neutrophil variability lead to some of the variation between experiments, though this should not be the cause of any differences within one assay. This could explain the differences in binding index and percentage binding between experiments, but not inter varietal differences within experiments, as var. *gattii* and var. *neoformans* were always run in parallel in each assay.

Sahu et al in a study of binding efficiency of metastable C3b to encapsulated cryptococci of all four serotypes showed a stepwise decrease in binding efficiency that parallels the serotype-dependent decrease in density of xylopyranosyl residues eg., C>B>A>D (Sahu et al. 1994). This is contrasted by evaluations of C3 accumulation on encapsulated cryptococci after incubation in normal human serum showing a greater rate of accumulation on cells of serotypes A and D than serotypes B and C (Washburn et al. 1991; Young and Kozel, 1993) These apparently

conflicting results illustrate the multifactorial nature of alternative pathway activation by the cryptococcal capsule. The study by Sahu et al considered a single component of the processs, but rate amplification is the end result of several steps of alternative pathway activation. It is therefore suggested that serotype-dependant variations in capsular structure may have multiple and independent effects on initiation, amplification and regulation of the alternative pathway (Kozel, 1996). Neutrophil-cryptococcal interactions are likely to be complex and serotype-dependant variations difficult to interpret clearly.

The assays comparing varying amounts of NHS at 30 and 120 minutes incubation showed that in the 30 minute assay binding was maximal when 100 μl NHS was used. At 120 minutes, although the percentage was near maximal when 100 μl NHS was used, the binding index rose considerably in all isolates and would appear not to have reached a maximum by 120 minutes. This could be because cryptococcal opsonisation was limited by the volume of available NHS, or despite optimal opsonisation, it may take many hours to allow full cryptococcal-neutrophil interaction to occur under the influence of secreted cytokines and altering configurations of complement receptors.

Young and Kozel showed that maximal binding of C3 fragments to all cryptococcal serotypes had occurred for some strains by 20 minutes and almost always by 40 minutes (Young and Kozel, 1993). Therefore the increase in percentage binding and

binding index with time may reflect simply a longer period following C3 binding to allow cryptococcal attachment to occur, but may also reflect neutrophil cell surface receptor activation and clustering, allowing more neutrophil-cryptococcal attachment. To investigate this further assays with greater quantities of NHS over longer neutrophil cryptococcal incubation periods could be done.

All the timed assays over 90-120 minutes showed that the percentage binding appeared to maximise by 60-90 minutes incubation. In most assays the binding index however had not plateaued by 120 minutes, which could reflect the ongoing interactions and changes at the neutrophil and cryptococcal surfaces. All neutrophils that could bind had done so by 90 minutes, but further binding with more yeasts continued to take place up to 120 minutes. In order to find the upper limit of the binding index longer incubation periods could be studied. The binding index curves do not appear to be beginning to plateau, so it is not possible to calculate from Michaelis-Menten kinetics the maximal binding, which must be limited by physical spacial factors if not by biological factors.

Altering the method of counting/staining from inverted coverslip to Giemsa staining may have lead to differences between experiments. It was assumed that although there may be a consistent difference between the two methods, this would not affect differences between isolates within the same experiment. For each set of experiments the same source of NHS was used, so this in itself should not lead to variation.

In view of earlier apparently consistent differences between the few isolates of the two varieties when studied with the coverslip method this was surprising. In the later Giemsa assays using a total of 4 strains at a time, handling of coverslips did become more awkward and may have contributed to variable results.

There is considerable variation in percentage binding and binding index between the different assays. This may be due to variations between and within the neutrophil donors used and even variable degrees of stain uptake leading to differences in the number of cryptococci counted as associated with each neutrophil.

In order to overcome these difficulties one could run direct comparisons of the two counting methodologies, or run more assays over a single condensed research period with the same neutrophil and normal human serum donors.

To differentiate better between external and internal yeasts more sophisticated labelling using rabbit anti-*C. neoformans* capsule antibody and goat anti-rabbit Ig-TRITC with visualisation by fluorescence could be used (Collins and Bancroft, 1992).

Using several isolates of each variety helps to clarify whether the behaviour of isolates of each variety is consistent, but to be able to make more valid comparisons between different assay conditions one would need to use similar isolates throughout.

Studies comparing different isolates of varying capsular size may not always be measuring the effect of capsular size. Other virulence factors such as melanin and mannitol production may also vary between such isolates. To confirm that any observed difference in virulence is due to capsular size differences for example, the differing effects should be shown to related to capsular polysaccharide alone. Another way to approach this is to measure alternative virulence factors simultaneously and demonstrate that they do not vary, or even better have strains in which only the gene controlling the virulence factor in question is absent and subsequent effects correlated with virulence (Kwon-Chung and Rhodes, 1986; Salas et al. 1996; Chang et al. 1996).

In conclusion, consistent differences in neutrophil binding to the two varieties of *C. neoformans* were not demonstrated. This may be because in the conditions used there are no differences between isolates, or perhaps because of methodological factors. Comparisons of binding results using both methods of counting staining would help to clarify this. It would also be of interest to look at binding at greater volumes of NHS to see if the binding index continues to rise to a maximum amount by 120 minutes incubation. Measurement of cytokine production in response to varying concentrations of cryptococci and even capsular polysaccharide and their effects on leucocyte chemotaxis, adherence and phagocytosis could also be investigated.

Ultimately, the definition of the gene(s) responsibe for serotype and varietal differences and the effect of adding and deleting these in vitro and in vivo outcomes would help elucidate the apparent differences in pathogenicity of the two varieties.

Chapter 7

A case of microscopy and latex antigen negative cryptococcal meningitis

7.1 Introduction

In the UK cryptococcal meningitis caused by *Cryptococcus neoformans* occurs in at least 4% of patients with AIDS, while in the USA 6-10% of cases are affected (Dismukes, 1988; Knight et al. 1993). In Edinburgh in 1995 there were 4 immunocompromised patients diagnosed with cryptococcosis of whom 3 were infected with HIV. In 1996 there were no new cases diagnosed, but one further HIV infected case was diagnosed in 1997. Headache is a common presenting symptom, but there are often no focal findings. The initial CSF findings may be virtually normal. A patient is reported in whom the initial diagnsosis of cryptococcal meningitis was only made after culture of CSF deposit. This highlights the importance of appropriate culture even where CSF findings are negative.

7.2 Case report

A 17 year old Caucasian male was diagnosed as HIV positive following routine screening during blood donation. He had previously been in a heterosexual relationship, but denied any other risk factors. On questioning at initial presentation he gave a 4 month history of mild intermittent headache associated with fever and night sweats. There was no history of travel, pigeon or antifungal exposure. Examination found him alert and orientated. His temperature was 39.8^{0}C, he had oral thrush and axillary lymphadenopathy without clinical hepatosplenomegaly. There were no signs of meningitis. Negative tests included: biochemistry and haematology profiles, repeated blood cultures, chest x-ray, induced sputum for *Pneumocystis carinii*, urinary bacterial culture and computerised tomography of the head. At presentation the CD4 lymphocyte count was $125/mm^3$, being 6% of the

total peripheral white blood cell count. At lumbar puncture the cerebrospinal fluid (CSF) protein was 474 mg/L (reference range 100 - 400 mg/L), glucose 2.8 mmol/L with no cells or organisms present, the pressure was not recorded. The concurrent serum glucose was 5.8 mmol/L. Gram staining of the CSF and cryptococcal antigen testing (IMMY -Immuno-mycologics, Norman, Okla) of CSF and blood were negative as was examination for other pathogens. After three days culture of the spun CSF deposit on 4% malt extract agar in air at 30^0C, four colonies of an apparently non-capsulated, relatively fluconazole resistant isolate (MIC 16μg/ml, confirmed at the PHLS Mycology Laboratory, Bristol) of *Cryptococcus neoformans* was cultured. A similar isolate was subsequently cultured from urine which then gave a positive antigen test at a 1:4 titre. Serotyping at the PHLS Reference Mycology Laboratory, Leeds showed the CSF isolate to be serotype A. The patient responded rapidly to amphotericin B preparations given for 3 weeks followed by oral itraconazole to which the isolate was sensitive in vitro. The latter was poorly tolerated and the patient continued on fluconazole 200 mg/d. He remained well while being prescribed fluconazole, antiretroviral therapy and pentamidine nebulisers until 19 months later. He then relapsed complaining of headache, vomiting and photophobia. At this stage he admitted that he had not been taking his fluconazole for the past 2-3 weeks. On this occasion CSF examination showed capsular cryptococci on India ink staining but was again otherwise acellular. The CSF glucose was 2.4 mmol/L, protein 1144 mg/L and serum glucose 7.9 mmol/L. Culture and latex antigen testing of CSF and peripheral blood were positive (antigen CSF titre 1:512) while the CD4 count was $0/mm^3$. On this admission a CSF opening pressure of 38 cm was recorded. 'Abelcet' (liposomal amphotericin) was

commenced and he was discharged after 3 weeks improving on oral itraconazole suspension. The isolate was reported to be more resistant to fluconazole on disc testing than the original isolate.

The original CSF isolate was grown in air at 37^0C and a neutrophil attachment assay carried out as described in Chapter 6. This showed that normal non heat inactivated human serum was necessary for attachment to occur. This implies that opsonisation by complement is required for attachment and therefore that some capsule is present. Further study of all isolates in the Regional Mycology Laboratory, Western General Hospital, Edinburgh from this patient grown at 37^0C in CSF showed significant capsules on India ink examination.

7.3 Discussion

The capsule of the yeast like fungus *Cryptococcus neoformans* varies in size depending on growth conditions (Vartivarian et al. 1993). In experimental mice the capsule size does not seem to affect virulence (Dykstra et al. 1977), although acapsular mutants are less virulent (Kwon-Chung and Rhodes, 1986). The capsule inhibits phagocytosis and may impair leukocyte migration (Kozel and Hermerath, 1984; Drouhet and Segretain, 1951), as well as being immunosuppressive (Khakpour and Murphy, 1987). Recent work suggests that capsular glucoronoxylomannan (GXM) induces interleukin-10 (a potent down regulator of proinflammatory cytokines) secretion by human monocytes (Vecchiarelli et al. 1996). Release of tumor necrosis factor α, interleukin-1β and interleukin-8, proinflammatory cytokines, by human neutrophils has also been correlated to

capsule size and quantity of GXM present by the same group of workers (Retini et al. 1996). There is a report that it may enhance HIV replication (Pettoello-Mantovani et al. 1992). Acapsular strains activate both classical and alternative complement pathways (Kozel et al. 1992), while capsular strains only activate the latter.

In one study 9% of AIDS patients had culture positive cryptococcal meningitis but negative CSF antigen detection tests although serum was negative in only 1% of patients (Chuck and Sande, 1989). Low levels of antigen, presence of immune-complexes (in non-protease treated serum samples), high titres (prozone effect) or poorly/non-encapsulated strains may contribute to such negative antigen tests (Currie et al. 1993; Sadamoto et al. 1993). In cases with extraneural cryptococcosis a much lower percentage of samples are positive. These factors and the possibility of a false-negative antigen test may not be commonly appreciated. Urinary screening is not always routinely carried out and urinary antigen testing not standardised although the prostate in particular may act as a reservoir.

Commercial kits to detect cryptococcal capsular antigen by latex agglutination and enzyme immunoassay are widely used, although there is some variation in performance (Tanner et al. 1994). The Immy kit used here has a sensitivity and specificity in serum of 97% and 93%, and in CSF of 93% and 93% respectively. This can be further improved without loss of sensitivity by raising the threshold for a positive result to 1:2 from the 1:1 indicated in the manufacturer's instructions. Pronase treatment of serum and CSF is used with this kit to limit non-specific

interference. All four test kits in this study were compared with culture of CSF (Tanner et al. 1994). The sensitivity of antigen detection in the CSF was 93-100% and specificity ranged from 93-98% while in serum sensitivity ranges were 83-97% and specificity ranges 95-100%. It must therefore be emphasised to clinicians and microbiologists that cryptococcal meningitis cannot be excluded simply on the basis of negative (even repeatedly so) CSF and serum antigen tests or microscopy. In this case the isolate was apparently acapsular on culture. It seems that this isolate could produce capsular material as shown by its serotyping result, behaviour in the neutrophil assay and subsequent in vitro culture in CSF. It may be that capsule formation was stimulated by the host and that initial antigen tests were negative due to low numbers of cryptococci present.

It is interesting to note that this patient was relatively well until 19 months after presentation and responded to further treatment. This is considerably longer than the median survival of 152 days in culture positive cases in the study reported by Chuck and Sande (Chuck and Sande, 1989). Improvement in antiretroviral therapy since that study was carried out may lead to a slower decline in immunity. Collins and Bancroft have shown that encapsulation of *C. neoformans* impaires antigen-specific T-cell responses, when compared with an acapsular mutant. (Collins and Bancroft, 1991). Therefore it may be that the lack of apparent capsule in this case also contributed to the longer time until relapse. Fluconazole resistance, though rarely documented in *C. neoformans*, may not correlate with clinical outcome. In this patient treatment was successful until compliance became a problem, although

the new isolate is capsular and more resistant than the original and the CD 4 count was 0/mm^3 at relapse. Reinfection with a different strain cannot be ruled out.

This case illustrates the importance of culture at 25-30 ^{0}C on an appropriate medium such as 4% malt extract agar in making the diagnosis of cryptococcosis. Standard 48 hour incubation as commonly carried out for more common bacterial pathogens is insufficient. Culture may be positive even where other parameters such as CSF cell count are normal. If the diagnosis is still elusive, repeated CSF examination should be considered. Such procedures should be carried out, particularly in immunosuppressed patients, even when antigen tests are negative.

Chapter 8

Conclusions and Further Work

8.1 Conclusions

These studies confirm that *Cryptococcus neoformans* is a significant pathogen in PNG. *C. neoformans* var. *gattii* causes disease frequently amongst apparently immunocompetent individuals. The increase in reported cases over the past two decades probably reflects increased clinical awareness and improved detection by latex antigen testing for cryptococcal capsular polysaccharide. The first cases of cryptococcal meningitis in HIV positive patients in PNG are reported - HIV will lead to a rise in cryptococcal meningitis in immunocompromised individuals. In PNG and elsewhere in the tropics cryptococcal meningitis is accompanied by significant morbidity and mortality. Frequent sequlae include blindness, deafness, cranial nerve palsies and death. This is perhaps due to late presentation as well as the effects of raised intracranial pressure.

The environmental source of *Cryptococcus neoformans* var. *gattii* has until recently been unclear. The finding of an association outwith PNG with *Eucalyptus camaldulensis,* lead to an investigation as to the likely source(s) of *C. neoformans* in PNG. It was found that of the species associated with *C. neoformans* var. *gattii* so far (*E. camaldulensis, E. tereticornis, E. grandis, E. rudis* and *E. blakelyi*), only *E. tereticornis* is endemic to PNG and few *E. camaldulensis* survive plantation trials. *E. camaldulensis* cannot be the main source of *C. neoformans* var. *gattii* in PNG. The known association of *C. neoformans* var. *gattii* with koalas and *C. neoformans* var. *neoformans* with avian guano lead to a search for possible mammalian and avian sources. Potential eucalypt, mammalian (particularly marsupial) and avian sources of *C. neoformans* were identified.

Many of these were sampled using similar methodology to that used by Ellis and Pfeiffer in their Australian eucalypt studies. Despite examination of 1100 samples, no source of *C. neoformans* was identified, suggesting that release is of very short duration, possibly from other as yet unexamined niches, as has been suggested in studies elsewhere in the tropics. More sensitive methods may be required to detect *C. neoformans* in this enivronment.

The epidemiology of cryptococcal meningitis in PNG was studied and a relatively high incidence of 33 cases /million population in Central Province and the National Capital District confirmed. Cases did occur amongst children, but the most frequent incidence was amongst immunocompetent males in their third decade of life. Clustering of cases appeared in the Rigo subprovince of Central Province. This suggests the focus for probable source or predisposition in this area. No seasonal correlation with rainfall could be identified.

Whether var. *gattii* meningitis in immunocompetent individuals is due to exposure to large amounts of var. *gattii*, virulence of var. *gattii* or occult immunodeficiency is unclear. No evidence in support of the latter hypothesis was found, and there is conflicting evidence for a difference in virulence between the two varieties. It is most likely that individuals are exposed to var. *gattii*, sometimes in large quantities, resulting in clinical disease.

Laboratory studies confirmed increased mucosity and capsule sizes of *C. neoformans* var. *gattii* as compared with var. *neoformans* on subculture. Investigation of human neutrophil-cryptococcal interactions did not demonstrate consistent differences between the two varieties.

A case of cryptococcal meningitis in Edinburgh illustrated the importance of extended culture in diagnosing cryptococcal meningitis where microscopy, including India ink staining, and antigen testing were negative. In PNG, where outwith Port Moresby not all CSF samples are cultured, undoubtedly cases may be missed. Initially it was thought that the isolate from this case was acapsulate, but neutrophil-cryptococcal studies in conjunction with serotyping and culture in CSF demonstrated the presence of capsule in all isolates from this patient. The availability of diagnostic and therapeutic options in Edinburgh may be contrasted with those in the Papua New Guinean setting.

8.2 Further research

Further study of cryptococcosis in PNG is possible. This includes typing of CSF isolates, full clinical and epidemiological documentation on prospectively studied patients. This information can be combined with well documented recent cases to highlight particular features of var. *gattii* meningitis such as prognostic indicators, visual loss and the effects of interventions such as corticosteroid use. Studies of cell mediated immunity, sero-epidemiology and HLA types are all feasible. Following extensive discussions some of these have been subsequently carried out by Dr Andrew Seaton, as alluded to in earlier chapters.

Environmental sampling could be extended to other potential sources and sampling intensified. More sensitive molecular techniques might be developed to detect *C. neoformans* in the environment. Typing of clinical isolates using established molecular techniques could be carried out to see whether clustering of cases is correlated with similar var. *gattii* isolates. These could also be compared with clinical and environmental isolates from elsewhere, particularly nearby Australia.

Laboratory studies comparing sensitivities of var. *gattii* and var. *neoformans* and their susceptibility to garlic may reveal therapeutically useful adjuncts to current treatment.

Further studies of *C. neoformans* var. *neoformans* and cellular deficiencies in particular are being carried out in laboratories worldwide, especially in North America and Australia. Fewer studies to date have attempted to compare and explain the apparent differences in host predispostition between var. *gattii* and var. *neoformans.*

In PNG currency fluctuations and cash shortages conspire with logistical and administrative difficulties to obstruct the supply of many basic drugs. In Africa HIV infection and AIDS have escalated rapidly; this pattern is likely to follow in PNG. The supply of cryptococcal latex antigen kits, amphotericin B and flucytosine is becoming increasingly irregular. Liposomal preparations, triazoles and antiretroviral drugs are not even generally available. Treatment of cryptococcal

meningitis is therefore becoming more difficult, despite improvements in therapy elsewhere.

Priority locally should be given to locate the environmental source(s) of *C. neoformans*, with the aim of reducing exposure and reducing the incidence of infection. There is substantial evidence that garlic may be of benefit as an adjunct in the treatment of cryptococcal meningitis. Garlic is widely available and cheap in the tropics and should be investigated clinically in this setting. For those who are infected, there is evidence that corticosteroid therapy reduces visual morbidity. It's use too should now be investigated to define the optimal dose.

Even these projects may well need some external support if they are to be carried out. Tuberculosis, malaria, HIV and childhood diseases such as diarrhoea, meningitis and respiratory tract infections are all high priorities. Among the adult population cardiovascular disease, diabetes mellitus and chronic obstructive pulmonary disease are emerging as significant problems. The overall burden of cryptococcal meningitis against this background is relatively low.

Answers to the issues raised here could result in effective low cost interventions to deal with cryptococcosis in PNG. *C. neoformans* var. *gattii* infection in immunocompetent individuals is of low priority outwith the tropics in the developed world (with the possible exception of Australia), yet cure of such patients is still a worthwhile and possible aim.

References

Ajello, L. (1958) Occurrence of *Cryptococcus neoformans* in soil. *Am J Hyg* **62,** 72-77.

Andrew, B. and Burton-Bradley, B.G. (1980) Psychosomatics of cryptococcosis. *PNG Med J* **23**, 51-53.

Anonymous (1965) Territory of Papua and New Guinea, Department of Agriculture, Stock and Fisheries: Annual Report, 1961-1963. Port Moresby.

Anonymous (1968) First Papua New Guinea Silvicultural Research Conference. Bulolo, PNG: Office of Forests, Papua New Guinea.

Anonymous (1980) Garlic in cryptococcal meningitis: a preliminary report of 21 cases. *Chinese Med J* **93**, 123-126.

Anonymous (1982) 1980 National Population Census. Port Moresby: National Statistical Office.

Anonymous (1993a) 1990 National Population Census. Port Moresby: National Statistical Office.

Anonymous (1993b) AIDS and HIV Quarterly Report No. 3. Port Moresby: STD/AIDS Unit, Papua New Guinea Department of Health.

Atkinson, A.J. Jr. and Bennett, J.E. (1968) Experience with a new skin test antigen prepared from *Cryptococcus neoformans. Am Rev Resp Dis* **97**, 637-643.

Balakrishnan, A.R. and Easwaran, K.R.K. (1993) CD and NMR studies on the aggregation of amphotericin-B in solution. *Biochim Biophys Acta* **1148,** 269-277.

Barone, J.A., Koh, J.G. and Bierman, R.H. (1993) Food interaction and steady-state pharmacokinetics of itraconazole capsules in healthy male volunteers. *Antimicrob Agents Chemother* **37,** 778-784.

Bauwens, L., Swinne, D., De Vroey, C. and De Meurichy, W. (1986) Isolation of *Cryptococcus neoformans* var. *neoformans* in the aviaries of the Antwerp Zoological Gardens. *Mykosen* **29**, 291-294.

Beehler, B. (1985) Conservation of New Guinea Rainforest Birds. In: Diamond, A.W. and Lovejoy, T.E. (Eds.) *Conservation of Tropical Forest Birds*, pp. 233-248. Norwich: Paston Press.

Beehler, B.M., Pratt, T.K. and Zimmerman, D.A. (1986) *Birds of New Guinea*, 1st edn. Princeton, New Jersey: Princeton University Press.

Begue, R.E. and Lindo-Soriano, I. (1991) Safety and efficacy of liposomal amphotericin B in treating AIDS-associated disseminated cryptococcosis. *J Infect Dis* **164,** 620-622.

Belay, T., Cherniak, R. and Shinoda, T. (1993) Specificity of *Cryptococcus neoformans* factor sera determined by enzyme-linked immunosorbent assay and dot enzyme assay. *Infect Immun* **61**, 2879-2885.

Benham, R.W. (1950) Cryptococcosis and blastomycosis. *Ann N Y Acad Sci* **50,** 1299-1314.

Bennett, J.E., Kwon-Chung, K.J. and Theodore, T.S. (1978) Biochemical differences between serotypes of *Cryptococcus neoformans*. *Sabouraudia* **16**, 167-174.

Bennett, J.E., Dismukes, W.E., Duma, R.J., Medoff, G., Sande, M.A., Gallis, H., Leonard, J., Fields, B.T., Bradshaw, M., Haywood, H., McGee, Z.A., Cate, T.R., Cobbs, C.G., Warner, J.F. and Alling, D.W. (1979) A comparison of amphotericin B alone and combined with flucytosine in the treatment of cryptococcal meningitis. *N Engl J Med* **301**, 126-131.

Bennett, J.E. (1995) Antifungal agents. In: Mandell, G.L., Bennett, J.E. and Dolin, R. (Eds.) *Principles and Practice of Infectious Diseases*, Fourth edn. pp. 401-410. New York: Churchill Livingstone.

Bennett, J.E. and Bailey, J.W. (1971) Control for rheumatoid factor in the latex test for cryptococcosis. *Am J Clin Pathol* **56**, 360-365.

Bhattacharjee, A.K., Bennett, J.E. and Glaudemans, C.P. (1984) Capsular polysaccharides of *Cryptococcus neoformans*. *Rev Infect Dis* **6**, 619-624.

Birley, H.D., Johnson, E.M., Mcdonald, P., Parry, C., Carey, P.B. and Warnock, D.W. (1995) Azole drug resistance as a cause of clinical relapse in AIDS patients with cryptococcal meningitis. *Int J STD AIDS* **6**, 353-355.

Block, E.R., Bennett, J.E. and Livoti, L.G. (1974) Flucytosine and amphotericin B: hemodyalisis effects on the plasma concentration and clearance. *Ann Int Med* **80,** 613.

Bloomfield, N., Gordon, M.A. and Elmendorf, D.F.J. (1963) Detection of *Cryptococcus neoformans* in body fluids by latex agglutination. *Proc Soc Exp Biol Med* **197,** 961-967.

Bodenhoff, J. (1968) Development of strains of *Cryptococcus neoformans* resistant to nystatin, amphotericin B, trichomycin and polymyxin B. *Acta Pathol Microbiol Scand* **73**, 572-582.

Boland, D.J. (1957) *Forest trees of Australia*, Melbourne: Thomas Nelson.

Bolanos, B. and Mitchell, T.G. (1989) Phagocytosis of *Cryptococcus neoformans* by rat alveolar macrophages. *J Med Vet Mycol* **27**, 203-217.

Bollinger, A. and Finckh, E.S. (1962) The prevalence of cryptococcosis in the koala *(Phascolarctos cinereus)*. *Med J Aust* **1**, 545-546.

Borgers, M. and Van de Ven, M.A. (1987) Degenerative changes in fungi after itraconazole treatment. *Rev Infect Dis* **9 S1**, S33-S42.

Braman, R.T. (1981) Cryptococcosis (torulosis) of prostate. *Urology* **17**, 284-285.

Branch, R.A. (1988) Prevention of amphotericin B-induced renal impairment. *Arch Int Med* **148,** 2389-2394.

Bruatto, M., Vidotto, V. and Maina, A.M. (1992) Growth of *Cryptococcus neoformans* in a thiamine-free medium. *Mycopathologia* **119**, 129-132.

Brueske, C.H. (1986) Proteolytic activity of a clinical isolate of *Cryptococcus neoformans*. *J Clin Microbiol* **23**, 631-633.

Bulmer, G.S. (1990) Twenty-five years with *Cryptococcus neoformans*. *Mycopathologia* **109**, 111-122.

Bulmer, G.S. and Sans, M.D. (1968) *Cryptococcus neoformans* III. Inhibition of phagocytosis. *J Bacteriol* **95**, 5-8.

Bulmer, G.S. and Tacker, J.R. (1975) Phagocytosis of *Cryptococcus neoformans* by alveolar macrophages. *Infect Immun* **11**, 73-79.

Buschke, A. (1894) Uber eine durch Coccicidien hervorgerufene Krankheit des Menschen. *Dtsch Med Wochenschr* **21,** 14.

Busse, O. (1894) Uber parasitare Zelleinshclusse und ihre Zuchtung. *Bakteriol* **16,** 175-180.

Cameron, M.L., Bartlett, J.A., Gallis, H.A. and Waskin, H.A. (1991) Manifestations of pulmonary cryptococcosis in patients with acquired immunodeficiency syndrome. *Rev Infect Dis* **13**, 64-67.

Campbell, C.H. and Arthur, R.K. (1964) A study of 2,000 admissions to the medical ward of the Port Moresby General Hospital. *Med J Aust* **1**, 989.

Campbell, G.D. (1966) Primary pulmonary cryptococcosis. *Am Rev Resp Dis* **94**, 236-243.

Casadevall, A., Mukherjee, J., Devi, S.J., Schneerson, R., Robbins, J.B. and Scharff, M.D. (1992) Antibodies elicited by a *Cryptococcus neoformans*-tetanus toxoid conjugate vaccine have the same specificity as those elicited in infection. *J Infect Dis* **165**, 1086-1093.

Casadevall, A., Spitzer, E.D., Webb, D. and Rinaldi, M.G. (1993) Susceptibilities of serial *Cryptococcus neoformans* isolates from patients with recurrent cryptococcal meningitis to amphotericin B and fluconazole. *Antimicrob Agents Chemother* **37**, 1383-1386.

Casscells, S.W. (1978) Aspergillus osteomyelitis of the tibia. *J Bone Joint Surg* **83**, 236-242.

Chakrabarti, A., Jatana, M., Kumar, P., Chatha, L., Kaushal, A. and Padhye, A.A. (1997) Isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus camaldulensis* in India. *J Clin Microbiol* **35**,3340-3342.

Champness, L.T. and Clezy, J.K.A. (1962) Torulosis in Papua and New Guinea. *Med J Aust* **1**, 560.

Chang, Y.C., Penoyer, L.A. and Kwon-Chung, K.J. (1996) The second capsule gene of *Cryptococcus neoformans*, CAP64, is essential for virulence. *Infect Immun* **64**, 1977-1983.

Chang, Y.C. and Kwon-Chung, K.J. (1994) Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol Cell Biol* **14**, 4912-4919.

Chanock, S.J., Toltzis, P. and Wilson, C. (1993) Cross-reactivity between *Stomatococcus mucilaginosus* and latex agglutination for cryptococcal antigen. *Lancet* **342**, 1119-1120.

Chaturvedi, V., Flynn, T., Niehaus, W.G. and Wong, B. (1996a) Stress tolerance and pathogenic potential of a mannitol mutant of *Cryptococcus neoformans*. *Microbiology* **142**, 937-943.

Chaturvedi, V., Wong, B. and Newman, S.L. (1996b) Oxidative killing of *Cryptococcus neoformans* by human neutrophils. Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates. *J Immunol* **156**, 3836-3840.

Chen, L.C., Pirofski, L.A. and Casadevall, A. (1997) Extracellular proteins of *Cryptococcus neoformans* and host antibody response. *Infect Immun* **65**, 2599-2605.

Chen, S.C., Muller, M., Zhou, J.Z., Wright, L.C. and Sorrell, T.C. (1997) Phospholipase activity in *Cryptococcus neoformans*: a new virulence factor? *J Infect Dis* **175**, 414-420.

Chen, S.C.A., Currie, B.J., Campbell, H.M., Fisher, D.A., Pfeiffer, T.J., Ellis, D.H. and Sorrell, T.C. (1997) *Cryptococcus neoformans* var. *gattii* infection in northern Australia: existence of an environmental source other than known host eucalypts. *Trans R Soc Trop Med Hyg* **91,** 547-550.

Cheong, W.Y., Thomas, A. and Tan, K.P. (1988) Pulmonary cryptococcosis. *Singapore Med J* **29**, 223-231.

Cherniak, R., Reiss, E. and Turner, S.H. (1982) A galactomannan antigen of *Cryptococcus neoformans* serotype A. *Carbohydr Res* **103**, 239-250.

Cherniak, R., Morris, L.C., Anderson, B.C. and Meyer, S.A. (1991) Facilitated isolation, purification, and analysis of glucuronoxylomannan of *Cryptococcus neoformans*. *Infect Immun* **59**, 59-64.

Cherniak, R., Morris, L.C., Meyer, S.A. and Mitchell, T.B. (1993) Glucuronoxylomannan of *Cryptococcus neoformans* obtained from patients with AIDS. *Carbohydr Res* **249**, 405-413.

Cherniak, R. and Sundstrom, J.B. (1994) Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. *Infect Immun* **62**, 1507-1512.

Chuck, S.L. and Sande, M.A. (1989) Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *N Engl J Med* **321**, 794-799.

Clark, R.A., Greer, D., Atkinson, W., Valainis, G.T. and Hyslop, N. (1990a) Spectrum of *Cryptococcus neoformans* infection in 68 patients infected with human immunodeficiency virus. *Rev Infect Dis* **12**, 768-777.

Clark, R.A., Greer, D.L., Valainis, G.T. and Hyslop, N.E. (1990b) *Cryptococcus neoformans* pulmonary infection in HIV-1-infected patients. *J Acq Imm Def Synd* **3**, 480-484.

Cleary, J.D., Taylor, J.W. and Chapman, S.W. (1992) Itraconazole in antifungal therapy. *Ann Pharmacoth* **26**, 502-509.

Coates, B.J. (1985) *The Birds of Papua New Guinea*, 1st edn. Alderley, Queensland: Dove Publications.

Cobcroft, R., Kronenberg, H. and Wilkinson, T. (1978) *Cryptococcus* in bagpipes. *Lancet* **1**, 1368-1369.

Cohen, J. (1982) The pathogenesis of cryptococcosis. *J Infect* **5**, 109-116.

Cohen, J., Perfect, J.R. and Durack, D.T. (1982) Cryptococcosis and the basidiospore. *Lancet* **1**, 1301

Collins, H.L. and Bancroft, G.J. (1991) Encapsulation of *Cryptococcus neoformans* impairs antigen-specific T-cell responses. *Infect Immun* **59**, 3883-3888.

Collins, H.L. and Bancroft, G.J. (1992) Cytokine enhancement of complement-dependent phagocytosis by macrophages: synergy of tumor necrosis factor-alpha and granulocyte-macrophage colony-stimulating factor for phagocytosis of *Cryptococcus neoformans*. *Eur J Immunol* **22**, 1447-1454.

Costermans, L. (1994) *Trees of Victoria and adjoining areas*, Fifth edn. Seaford, Victoria: Rolls Printing.

Cox, L.B. and Tolhurst, J.C. (1946) *Human torulosis, a clinical pathological and microbiological study with a report of 13 cases.* Melbourne: Melbourne University Press.

Crampin, A.C., Matthews, R.C., Hall, D. and Evans, E.G.V. (1993) PCR fingerprinting of *Cryptococcus neoformans* by random amplificatin of polymorphic DNA. *J Med Vet Mycol* **31**, 463-465.

Crane, J.K. and Shih, H. (1993) Syncope and cardiac arrhythmia due to an interaction between itraconazole and terfenadine. *Am J Med* **95,** 445-446.

Craven, P.C., Ludden, T.M. and Drutz, D.J. (1979) Excretion pathways of amphotericin B. *J Infect Dis* **140,** 329.

Crowe, S.M., Carlin, J.B., Stewart, K.I., Lucas, C.R. and Hoy, J.F. (1991) Predictive value of CD4 lymphocyte numbers for the development of opportunistic infections and malignancies in HIV-infected persons. *J Acq Imm Def Synd* **4,** 770-776.

Currie, B., Vigus, T., Leach, G. and Dwyer, B. (1990) *Cryptococus neoformans* var. *gattii. Lancet* **336**, 1442.

Currie, B.P., Freundlich, L.F., Soto, M.A. and Casadevall, A. (1993) False-negative cerebrospinal fluid cryptococcal latex agglutination tests for patients with culture-positive cryptococcal meningitis. *J Clin Microbiol* **31**, 2519-2522.

Currie, B.P., Freundlich, L.F. and Casadevall, A. (1994) Restriction fragment length polymorphism analysis of *Cryptococcus neoformans* isolates from environmental (pigeon excreta) and clinical sources in New York City. *J Clin Microbiol* **32**, 1188-1192.

Dalgleish, A.G. (1981) Concurrent hydatid disease and cryptococcosis in a 16-year-old girl. *Med J Aust* **2**, 144-145.

Davis, L.E., Shen, J.K. and Cai, Y. (1990) Antifungal activity in human cerebrospinal fluid and plasma after intravenous administration of Allium sativum. *Antimicrob Agents Chemother* **34**, 651-653.

De Wytt, C.N., Dickson, P.L. and Holt, G.W. (1982) Cryptococcal meningitis. A review of 32 years experience. *J Neurol Sci* **53**, 283-292.

Delfino, D., Cianci, L., Lupis, E., Celeste, A., Petrelli, M.L., Curro, F., Cusumano, V. and Teti, G. (1997) Interleukin-6 production by human monocytes stimulated with *Cryptococcus neoformans* components. *Infect Immun* **65**, 2454-2456.

Denning, D.W., Tucker, R.M., Hanson, L.H., Hamilton, J.R. and Stevens, D.A. (1989) Itraconazole therapy for cryptococcal meningitis and cryptococcosis. *Arch Int Med* **149**, 2301-2308.

Denning, D.W., Stevens, D.A. and Hamilton, J.R. (1990) Comparison of Guizotia abyssinica seed extract (birdseed) agar with conventional media for selective identification of *Cryptococcus neoformans* in patients with acquired immunodeficiency syndrome. *J Clin Microbiol* **28**, 2565-2567.

Denning, D.W., Armstrong, R.W., Fishman, M. and Stevens, D.A. (1991a) Endophthalmitis in a patient with disseminated cryptococcosis and AIDS who was treated with itraconazole. *Rev Infect Dis* **13**, 1126-1130.

Denning, D.W., Armstrong, R.W., Lewis, B.H. and Stevens, D.A. (1991b) Elevated cerebrospinal fluid pressures in patients with cryptococcal meningitis and acquired immunodeficiency syndrome. *Am J Med* **91**, 267-272.

Detmers, P.A., Wright, S.D., Olsen, E., Kimball, B. and Cohn, Z.A. (1987) Aggregation of complement receptors on human neutrophils in the absence of ligand. *J Cell Biol* **105,** 1137-1145.

Devi, S.J., Schneerson, R., Egan, W., Ulrich, T.J., Bryla, D., Robbins, J.B. and Bennett, J.E. (1991) *Cryptococcus neoformans* serotype A glucuronoxylomannan-protein conjugate vaccines: synthesis, characterization, and immunogenicity. *Infect Immun* **59**, 3700-3707.

Diamond, R.D., Root, R.K. and Bennett, J.E. (1972) Factors influencing killing of *Cryptococcus neoformans* by human leukocytes in vitro. *J Infect Dis* **125**, 367-376.

Diamond, R.D. (1995) *Cryptococcus neoformans*. In: Mandell, G.L., Bennett, J.E., Dolin, R.(Eds.) *Principles and Practice of Infectious Diseases*, 4th edn. pp. 2331-240. New York: Churchill Livingstone.

Diamond, R.D. and Bennett, J.E. (1974) Prognostic factors in cryptococcal meningitis. A study in 111 cases. *Ann Int Med* **80**, 176-181.

Diamond, R.D. and Erickson, N.F. (1982) Chemotaxis of human neutrophils and monocytes induced by *Cryptococcus neoformans*. *Infect Immun* **38**, 380-382.

Diasio, R.B., Bennett, J.E. and Myers, C.E. (1978) Mode of action of 5-fluorocytosine. *Biochem Pharmacol* **27,** 703.

Dismukes, W.E., Cloud, G.A. and Gallis, H.A. (1983) Treatment of systemic mycoses with ketoconazole: emphasis on toxicity and clinical response in 52 patients. *Ann Int Med* **98,** 13-20.

Dismukes, W.E., Cloud, G., Gallis, H.A., Kerkering, T.M., Dedoff, G., Craven, P.C., Kaplowitz, L.G., Fisher, J.F., Gregg, C.R., Bowles, C.A. and et al (1987) Treatment of cryptococcal meningitis with combination amphotericin B and flucytosine for four as compared with six weeks. *N Engl J Med* **317**, 334-341.

Dismukes, W.E. (1988) Cryptococcal meningitis in patients with AIDS. *J Infect Dis* **157**, 624-628.

Dismukes, W.E., Bradsher Jr, R.W. and Cloud, G.C. (1992) Itraconazole therapy for blastomycosis and histoplasmosis. *Am J Med* **93,** 489-497.

Doft, B.H. and Curtin, V.T. (1982) Combined ocular infection with cytomegalovirus and cryptococcosis. *Arch Ophthalmol* **100**, 1800-1803.

Dromer, F., Gueho, E., Ronin, O. and Dupont, B. (1993) Serotyping of *Cryptococcus neoformans* by using a monoclonal antibody specific for capsular polysaccharide. *J Clin Microbiol* **31**, 359-363.

Dromer, F., Mathoulin, S., Dupont, B., Letenneur, L. and Ronin, O. (1996) Individual and environmental factors associated with infection due to *Cryptococcus neoformans* serotype D. French Cryptococcosis Study Group. *Clin Infect Dis* **23**, 91-96.

Drouhet, E. and Segretain, G. (1951) Inhibition de la migration leucocytaire in vitro par un polyoside capsulaire de *Torulopsis (Cryptococcus) neoformans.. Annal Inst Pasteur* **81**, 674-676.

Duncan, R.A., von Reyn, C.F., Alliegro, G.M., Toossi, Z., Sugar, A.M. and Levitz, S.M. (1993) Idiopathic CD4+ T-lymphocytopenia--four patients with opportunistic infections and no evidence of HIV infection. *N Engl J Med* **328**, 393-398.

Dykstra, M.A., Friedman, L. and Murphy, J.W. (1977) Capsule size of *Cryptococcus neoformans*: Control and relation to virulence. *Infect Immun* **16**, 129-135.

Ellis, D., Pfeiffer, T., Ruma, P., Brownlee, A., Chen, S. and Sorrell, T. (1996) Distribution and frequency of *C.neoformans* var. *gattii* among the species of *Eucalyptus. 3rd International Conference on Cryptococcus and Cryptococcosis.* September Paris 47-48. (Abstract)

Ellis, D. and Pfeiffer, T. (1992) The ecology of *Cryptococcus neoformans. EurJ Epidemiol* **8**, 321-325.

Ellis, D.H. and Pfeiffer, T.J. (1990a) Ecology, life cycle, and infectious propagule of *Cryptococcus neoformans. Lancet* **336**, 923-925.

Ellis, D.H. and Pfeiffer, T.J. (1990b) Natural habitat of *Cryptococcus neoformans* var. *gattii. J Clin Microbiol* **28**, 1642-1644.

Emmons, C.W. (1951) Isolation of *Cryptococcus neoformans* from soil. *J Bacteriol* **62,** 685-690.

Emmons, C.W. (1955) Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (*Columbia livia). Am J Hyg* **62**, 227.

Eng, R.H., Bishburg, E., Smith, S.M. and Kapila, R. (1986) Cryptococcal infections in patients with acquired immune deficiency syndrome. *Am J Med* **81**, 19-23.

Erke, K.H. and Schneidau, J.D., Jr. (1973) Relationship of some *Cryptococcus neoformans* hypha-forming strains to standard strains and to other species of yeasts as determined by deoxyribonucleic acid base ratios and homologies. *Infect Immun* **7**, 941-948.

Espinel-Ingroff, A., Kish, C.W., Jr., Kerkering, T.M., Fromtling, R.A., Bartizal, K., Galgiani, J.N., Villareal, K., Pfaller, M.A., Gerarden, T., Rinaldi, M.G. and et al (1992) Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. *J Clin Microbiol* **30**, 3138-3145.

Evans, E.E. (1949) An immunologic comparison of twelve strains of *Cryptococcus neoformans (Torula histolytica). Proc Soc Exp Biol Med* **71,** 644-646.

Farmer, S.G. and Komorowski, R.A. (1973) Histologic response to capsule-deficient *Cryptococcus neoformans. Arch Pathol* **96**, 383-387.

Fisher, D., Burrow, J., Lo, D. and Currie, B. (1993) *Cryptococcus neoformans* in tropical northern Australia: predominantly variant *gattii* with good outcomes. *Aust N Z J Med* **23**, 678-682.

Flannery, T.F. (1990) *The Mammals of New Guinea*, Carina, Qld: Robert Brown & Associates.

Frank, U.K., Nishimura, S.L., Li, N.C., Sugai, K., Yajko, D.M., Hadley, W.K. and Ng, V.L. (1993) Evaluation of an enzyme immunoassay for detection of cryptococcal capsular polysaccharide antigen in serum and cerebrospinal fluid. *J Clin Microbiol* **31**, 97-101.

Freeman, W. (1931) Torula infection of the central nervous system. *J Psychol fur Neurol* **43** 236-345.

Frey, D. and Durie, E.B. (1964) The isolation of *Cryptococcus neoformans (Torula histolytica)* from soil in New Guinea and pigeon droppings in Sydney, New South Wales. *Med J Aust* **1**, 947.

Frey, D. and Durie, E.B. (1970) Deep mycoses reported from Australia and New Guinea during the years 1956 to 1969. *Med J Aust* **2**, 1117-1123.

Fromtling, R.A., Abruzzo, G.K. and Bulmer, G.S. (1986) *Cryptococcus neoformans*: comparisons of in vitro antifungal susceptibilities of serotypes AD and BC. *Mycopathologia* **94**, 27-30.

Fromtling, R.A. and Bulmer, G.S. (1978) In vitro effect of aqueous extract of garlic (Allium sativum) on the growth and viability of *Cryptococcus neoformans*. *Mycologia* **70**, 397-405.

Frothingham, L. (1902) A tumor-like lesion in the lung of a horse caused by a blastomyces (torula). *J Med Res* **3,** 31-43.

Gadebusch, H.H. (1972) Mechanisms of native and acquired resistance to infection with *Cryptococcus neoformans*. *CRC Crit Rev Microbiol* **1**, 311-320.

Garrity, J.A., Herman, D.C., Imes, R., Fries, P., Hughes, C.F. and Campbell, R.J. (1993) Optic nerve sheath decompression for visual loss in patients with acquired immunodeficiency syndrome and cryptococcal meningitis with papilledema. *Am J Ophthalmol* **116**, 472-478.

Ghannoum, M.A., Ibrahim, A.S., Fu, Y., Shafiq, M.C., Edwards, J.E., Jr. and Criddle, R.S. (1992) Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. *J Clin Microbiol* **30**, 2881-2886.

Glaser, J.B. and Garden, A. (1985) Inoculation of cryptococcosis without transmission of the acquired immunodeficiency syndrome *N Engl J Med* **313**, 266.

Goldner, M., Farkas-Himsley, H., Kormendy, A. and Skinner, M. (1983) Bacterial phagocytosis monitored by fluorescence and extracellular quenching: ingestion and intracellular killing. *Lab Med* **14**, 291-294.

Gordon, M.A. and Casadevall, A. (1995) Serum therapy for Cryptococcal meningitis *Clin Infect Dis* **21**, 1477-1479.

Goren, M.B. and Middlebrook, G.M. (1967) Protein conjugates of polysaccharide from *Cryptococcus* neoforman. *J Immunol* **98**, 901-913.

Gould, P.R. and Gould, I.M. (1985) Cryptococcosis in Zimbabwe. *Trans R Soc Trop Med Hyg* **79**, 67-69.

Graham, I.L., Gresham, H.D. and Brown, E.J. (1989) An immobile subset of plasma membrane CD11b/CD18 (Mac-1) is involved in phagocytosis of targets recognized by multiple receptors. *J Immunol* **142,** 2352-2358.

Granger, D.L., Perfect, J.R. and Durack, D.T. (1985) Virulence of *Cryptococcus neoformans*. Regulation of capsule synthesis by carbon dioxide. *J Clin Invest* **76**, 508-516.

Graybill, J.R. and Alford, R.H. (1974) Cell-mediated immunity in Cryptococcosis. *Cell Immunol* **14**, 12-21.

Groube, L.J., Chappell, J., Muke, J. and Price, D. (1986) A 40,000 year-old human occupation site at Huon Peninsula, Papua New Guinea. *Nature* **324**, 353-355.

Hageage, G.J. and Harrington, B.J. (1984) Use of calcofluor white in clinical mycology. *Lab Med* **15,** 109-112.

Hall, L.S. (1983) Black Flying Fox. In: Strahan, R. (Ed.) *The Australian Museum Complete Book of Australian Mammals*, pp. 282-283. Sydney: Angus & Robertson.

Hamilton, A.J. and Goodley, J. (1993) Purification of the 115-kilodalton exoantigen of *Cryptococcus neoformans* and its recognition by immune sera. *J Clin Microbiol* **31**, 335-339.

Hammerman, K.J., Powell, K.E., Christianson, C.S., Huggin, P.M., Larsh, H.W., Vivas, J.R. and Tosh, F.E. (1973) Pulmonary cryptococcosis: clinical forms and treatment. A Center for Disease Control cooperative mycoses study. *Am Rev Resp Dis* **108**, 1116-1123.

Hay, R.J., Mackenzie, D.W., Campbell, C.K. and Philpot, C.M. (1980) Cryptococcosis in the United Kingdom and the Irish Republic: an analysis of 69 cases. *J Infect* **2**, 13-22.

Haynes, K.A., Sullivan, D.J., Coleman, D.C., Clarke, J.C.K., Emilianus, R., Atkinson, C. and Cann, K.J. (1995) Involvement of multiple *Cryptococcus neoformans* strains in a single episode of cryptococcosis and reinfection with novel strains in recurrent infection demonstrated by random amplification of polymorphic DNA and DNA fingerpriniting. *J Clin Microbiol* **33**, 99-102.

Henderson, D.K., Edwards, J.E., Dismukes, W.E. and Bennett, J.E. (1981) Meningitis produced by different serotypes of *Cryptococcus neoformans. Annual Meeting of the American Society of Microbiology* 315 (Abstract).

Henderson, D.K., Bennett, J.E. and Huber, M.A. (1982) Long-lasting, specific immunologic unresponsiveness associated with cryptococcal meningitis. *J Clin Invest* **69**, 1185-1190.

Henry, S.R. and Suckling, G. (1985) A review of the ecology of the Sugar Glider (*Petaurus breviceps*). In: Smith, A. and Hume, A. (Eds.) *Possums and Gliders*, pp. 355-388. Chipping Norton, NSW: Surrey Beatty & Sons.

Heyligers, P.C. (1982) Semi-deciduous scrub and forest and Eucalypt woodland in the Port Moresby area. In: Gressit, J.L. (Ed.) *Monographiae Biologicae, Vol 42* pp. 437-457. The Hague: Dr. W. Junk.

Hill, J.O. and Aguirre, K.M. (1994) CD4+ T cell-dependent acquired state of immunity that protects the brain against *Cryptococcus neoformans*. *J Immunol* **152**, 2344-2350.

Horgan, M., Boothman, S., Keane, C.T., Temperley, I.J., Mulcahy, F. and Jackson, F. (1990) Cryptococcal meningitis occurring in HIV infected individuals. *Irish Med J* **83**, 157-158.

Horn, C.A. and Washburn, R.G. (1995) Anticryptococcal activity of NK cell-enriched peripheral blood lymphocytes from human immunodeficiency virus-infected subjects: responses to interleukin-2, interferon-gamma, and interleukin-12. *J Infect Dis* **172**, 1023-1027.

Hostetler, J.S., Clemons, K.V., Hanson, L.H. and Stevens, D.A. (1992) Efficacy and safety of amphotericin B colloidal dispersion compared with those of amphotericin B deoxycholate suspension for treatment of disseminated murine cryptococcosis. *Antimicrob Agents Chemother* **36**, 2656-2660.

Hsu, S. and Burnett, R.R. (1993) The effect of amphotericin B on the K channel activity of MDCK cells. *Biochim Biophys Acta* **1152,** 189-191.

Huffnagle, G.B., Chen, G.H., Curtis, J.L., McDonald, R.A., Strieter, R.M. and Toews, G.B. (1995) Down-regulation of the afferent phase of T cell-mediated pulmonary inflammation and immunity by a high melanin-producing strain of *Cryptococcus neoformans*. *J Immunol* **155**, 3507-3516.

Hutchinson, D.O., Anderson, N.E., Ingram, R.J., Thomas, M.G., Ellis-Pegler, R.B., Bremner, D.A. and Parr, D.H. (1991) Cryptococcal meningitis in Auckland 1969-89. *N Z Med J* **104**, 57-59.

Igel, H.J. and Bolande, R.P. (1966) Humoral defense mechanisms in cryptococcosis: substances in normal human serum, saliva, and cerebrospinal fluid affecting the growth of *Cryptococcus neoformans*. *J Infect Dis* **116**, 75-83.

Ishaq, C.M., Bulmer, G.S. and Felton, F.G. (1968) An evaluation of various environmental factors affecting the propagation of *Cryptococcus* neoformas. *Mycopathol Mycol Appl* **35**, 81-90.

Iwata, K., Yamashita, T., Ohsumi, M., Baba, M., Naito, N., Taki, A. and Yamada, N. (1990) Comparative morphological and biological studies on the itraconazole- and ketoconazole-resistant mutants of *Cryptococcus neoformans*. *J Med Vet Mycol* **28**, 77-90.

Jack, R.M., Ezzell, R.M., Hartwig, J. and Fearon, D.T. (1986) Differential interaction of the C3b/C4b receptor and MHC class I with the cytoskeleton of human neutrophils. *J Immunol* **137,** 3996-4003.

Jacobs, M.R. (1979) *Eucalypts for Planting.* Food and Agriculture Organisation Forestry Series. Rome: United Nations.

Jacobson, E.S., Jenkins, N.D. and Todd, J.M. (1994) Relationship between superoxide dismutase and melanin in a pathogenic fungus. *Infect Immun* **62**, 4085-4086.

Jacobson, E.S. and Tinnell, S.B. (1993) Antioxidant function of fungal melanin. *J Bacteriol* **175**, 7102-7104.

Jacobson, E.S. and Vartivarian, S.E. (1992) Iron assimilation in *Cryptococcus neoformans*. *J Med Vet Mycol* **30**, 443-450.

Jennings, T.S. and Hardin, T.C. (1993) Treatment of aspergillosis with itraconazole. *Ann Pharmacoth* **27,** 1206-1211.

Johnston, S.R., Corbett, E.L., Foster, O., Ash, S. and Cohen, J. (1992) Raised intracranial pressure and visual complications in AIDS patients with cryptococcal meningitis. *J Infect* **24**, 185-189.

Joly, V., Geoffray, C., Reynes, J., Goujard, C., Mechali, D., Maslo, C., Raffi, F. and Yeni, P. (1996) Amphotericin B in a lipid emulsion for the treatment of cryptococcal meningitis in AIDS patients. *J Antimicrob Chemother* **38**, 117-126.

Just-Nubling, G. (1996) Initial triple combination in AIDS patients with cryptococcal meningitis. *3rd International Conference on Cryptococcus and Cryptococcosis.* Paris, France, 103 (Abstract).

Kapend'a, K., Komichelo, K., Swinne, D. and Vandepitte, J. (1987) Meningitis due to *Cryptococcus neoformans* biovar *gattii* in a Zairean AIDS patient. *Eur J Clin Microbiol* **6**, 320-321.

Kariks, J. (1967) Cryptococcosis: solitary pulmonary and multiple cerebral tumours. *Med J Aust* **1**, 651-652.

Kerkering, T.M., Duma, R.J. and Shadomy, S. (1981) The evolution of pulmonary cryptococcosis: clinical implications from a study of 41 patients with and without compromising host factors. *Ann Int Med* **94**, 611-616.

Khakpour, F.R. and Murphy, J.W. (1987) Characterization of a third-order suppressor T cell (Ts3) induced by cryptococcal antigen(s). *Infect Immun* **55**, 1657-1662.

Khoury, M.B., Godwin, J.D., Ravin, C.E., Gallis, H.A., Halvorsen, R.A. and Putman, C.E. (1984) Thoracic cryptococcosis: immunologic competence and radiologic appearance. *Am J Roent* **142**, 893-896.

Klein, E. (1901) Pathogenic microbes in milk. *J Hyg* **1**, 78-95.

Knight, F.R., Mackenzie, D.W., Evans, B.G., Porter, K., Barrett, N.J. and White, G.C. (1993) Increasing incidence of cryptococcosis in the United Kingdom. *J Infect* **27**, 185-191.

Kovacs, J.A., Kovacs, A.A., Polis, M., Wright, W.C., Gill, V.J., Tuazon, C.U., Gelmann, E.P., Lane, H.C., Longfield, R., Overturf, G. and et al (1985) Cryptococcosis in the acquired immunodeficiency syndrome. *Ann Int Med* **103**, 533-538.

Kozel, T.R., Highison, B. and Stratton, C.J. (1984) Localization on encapsulated *Cryptococcus neoformans* of serum components opsonic for phagocytosis by macrophages and neutrophils. *Infect Immun* **43**, 574-579.

Kozel, T.R., Pfrommer, G.S. and Redelman, D. (1987) Activated neutrophils exhibit enhanced phagocytosis of *Cryptococcus neoformans* opsonized with normal human serum. *Clin Exp Immunol* **70**, 238-246.

Kozel, T.R., Wilson, M.A. and Murphy, J.W. (1991) Early events in initiation of alternative complement pathway activation by the capsule of *Cryptococcus neoformans*. *Infect Immun* **59**, 3101-3110.

Kozel, T.R., Wilson, M.A. and Welch, W.H. (1992) Kinetic analysis of the amplification phase for activation and binding of C3 to encapsulated and nonencapsulated *Cryptococcus neoformans*. *Infect Immun* **60**, 3122-3127.

Kozel, T.R. (1993) Activation of the complement system by the capsule of *Cryptococcus neoformans*. *Curr Top Med Mycol* **5**, 1-26.

Kozel, T.R. (1996) Activation of the complement system by pathogenic fungi. *Clin Microbiol Rev* **9**, 34-46.

Kozel, T.R., Tabuni, A., Young, B.J. and Levitz, S.M. (1996) Influence of opsonization conditions on C3 deposition and phagocyte binding of large- and small-capsule *Cryptococcus neoformans* cells. *Infect Immun* **64**, 2336-2338.

Kozel, T.R. and Hermerath, C.A. (1984) Binding of cryptococcal polysaccharide to *Cryptococcus neoformans*. *Infect Immun* **43**, 879-886.

Kupfer, C. and McCrane, E. (1974) A possible cause of decreased vision in cryptococcal meningitis. *Investig Ophthalmol* **13**, 801-804.

Kwon-Chung, K.J. (1975) A new genus, filobasidiella, the perfect state of *Cryptococcus neoformans*. *Mycologia* **67**, 1197-1200.

Kwon-Chung, K.J., Hill, W.B. and Bennett, J.E. (1981) New, special stain for histopathological diagnosis of cryptococcosis. *J Clin Microbiol* **13**, 383-387.

Kwon-Chung, K.J., Bennett, J.E. and Rhodes, J.C. (1982a) Taxonomic studies on Filobasidiella species and their anamorphs. *Antonie van Leeuwenhoek* **48**, 25-38.

Kwon-Chung, K.J., Polacheck, I. and Bennett, J.E. (1982b) Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). *J Clin Microbiol* **15**, 535-537.

Kwon-Chung, K.J., Varma, A. and Howard, D.H. (1990) Ecology of *Cryptococcus neoformans* and prevalence of its two varieties in AIDS and non-AIDS associated Cryptococcosis. In: Vanden Bossche, H. and et al, (Eds.) *Mycoses in AIDS Patients*, pp. 103-113. New York: Plenum Press.

Kwon-Chung, K.J., Edman, J.C. and Wickes, B.L. (1992a) Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect Immun* **60**, 602-605.

Kwon-Chung, K.J., Wickes, B.L., Stockman, L., Roberts, G.D., Ellis, D. and Howard, D.H. (1992b) Virulence, serotype, and molecular characteristics of environmental strains of *Cryptococcus neoformans* var. *gattii*. *Infect Immun* **60**, 1869-1874.

Kwon-Chung, K.J. and Bennett, J.E. (1984) Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *Am J Epidemiol* **120**, 123-130.

Kwon-Chung, K.J. and Bennett, J.E. (Eds.) (1992) *Medical Mycology.* pp. 397-446. Malvern, Pa: Lea & Febiger.

Kwon-Chung, K.J. and Rhodes, J.C. (1986) Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans. Infect Immun* **51**, 218-223.

Lalloo, D., Fisher, D., Naraqi, S., Laurenson, I., Temu, P., Sinha, A., Saweri, A. and Mavo, B. (1994) Cryptococcal meningitis (*C. neoformans* var. *gattii)* leading to blindness in previously healthy Melanesian adults in Papua New Guinea. *Q J Med* **87**, 343-349.

Larsen, R.A., Bozzette, S., McCutchan, J.A., Chiu, J., Leal, M.A. and Richman, D.D. (1989) Persistent *Cryptococcus neoformans* infection of the prostate after successful treatment of meningitis. California Collaborative Treatment Group. *Ann Int Med* **111**, 125-128.

Larsen, R.A., Bozette, S.A., Jones, B.E. and et al (1994) Fluconazole combined with flucytosine for treatment of cryptococcal meninigitis in patients with AIDS. *Clin Infect Dis* **19**, 741-745.

Laxalt, K.A. and Kozel, T.R. (1979) Chemotaxigenesis and activation of the alternative complement pathway by encapsulated and non-encapsulated *Cryptococcus neoformans. Infect Immun* **26**, 435-440.

Lazera, M.S., Wanke, B. and Nishikawa, M.M. (1993) Isolation of both varieties of *Cryptococcus neoformans* from saprophytic sources in the city of Rio de Janiero, Brazil. *J Med Vet Mycol* **31**, 449-454.

Lazera, M.S., Pires, F.D., Camillo-Coura, L., Nishikawa, M.M., Bezerra, C.C., Trilles, L. and Wanke, B. (1996) Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J Med Vet Mycol* **34**, 127-131.

Lazera, M.S., Cavalcanti, M.A.S., Trilles, L., Nishikawa, M.M., and Wanke, B. (1998) *Cryptococcus neoformans* var. *gattii* - evidence for a natural habitat related to decaying wood in a pottery tree hollow. *J Vet Med Mycol* **36**, 119-122

Lee, S.C., Dickson, D.W., Brosnan, C.F. and Casadevall, A. (1994) Human astrocytes inhibit *Cryptococcus neoformans* growth by a nitric oxide-mediated mechanism. *J Exp Med* **180**, 365-369.

Leenders, A.C., Reiss, P., Portegies, P., Clezy, K., Hop, W.C., Hoy, J., Borleffs, J.C., Allworth, T., Kauffmann, R.H., Jones, P., Kroon, F.P., Verbrugh, H.A. and de, M.S. (1997) Liposomal amphotericin B (AmBisome) compared with amphotericin B both followed by oral fluconazole in the treatment of AIDS-associated cryptococcal meningitis. *AIDS* **11**, 1463-1471.

Lehmann, P.F., Morgan, R.J. and Freimer, E.H. (1984) Infection with *Cryptococcus neoformans* var. *gattii* leading to a pulmonary cryptococcoma and meningitis. *J Infect* **9**, 301-306.

Levin, E. (1937) Torula infection of the central nervous system. *Arch Int Med* **59,** 667-684.

Levinson, D.J., Silcox, D.C., Rippon, J.W. and Thomsen, S. (1974) Septic arthritis due to nonencapsulated *Cryptococcus neoformans* with coexisting sarcoidosis. *Arthritis Rheum* **17**, 1037-1047.

Levitz, S.M., Farrell, T.P. and Maziarz, R.T. (1991) Killing of *Cryptococcus neoformans* by human peripheral blood mononuclear cells stimulated in culture. *J Infect Dis* **163**, 1108-1113.

Levitz, S.M., Tabuni, A., Wagner, R., Kornfeld, H. and Smail, E.H. (1992) Binding of unopsonized *Cryptococcus neoformans* by human bronchoalveolar macrophages: inhibition by a large-molecular-size serum component. *J Infect Dis* **166**, 866-873.

Levitz, S.M. and Tabuni, A. (1991) Binding of *Cryptococcus neoformans* by human cultured macrophages. Requirements for multiple complement receptors and actin. *J Clin Invest* **87**, 528-535.

Lewis, J.L. and Rabinovich, S. (1972) The wide spectrum of cryptococcal infections. *Am J Med* **53**, 315-322.

Lim, S.G., Sawyer, A.M. and Sercombe, J. (1993) Short report: The absorption of fluconazole and itraconazole under conditions of low intragastric acidity. *Aliment Pharmacol Ther* **7,** 317-321.

Lipson, B.K., Freeman, W.R., Beniz, J., Goldbaum, M.H., Hesselink, J.R., Weinreb, R.N. and Sadun, A.A. (1989) Optic neuropathy associated with cryptococcal arachnoiditis in AIDS patients. *Am J Ophthalmol* **107**, 523-527.

Littman, M.L. (1958) Capsule synthesis by *Cryptococus neoformans*. *N Y Acad Sci* **20** 623-648.

Lo, D. (1976) Cryptococcosis in the Northern Territory. *Med J Aust* **2**, 825-828.

Lo, P.C., Pernice, I., Gallo, M., Barbara, C., Luck, F.T., Criseo, G. and Pernice, A. (1997) Genetic relatedness and diversity of *Cryptococcus neoformans* strains in the Maltese Islands. *J Clin Microbiol* **35**, 751-755.

Loerinc, A.M., Bottone, E.J., Finkel, L.J. and Teirstein, A.S. (1988) Primary cryptococcal pneumonia mimicking Pneumocystis carinii pneumonia in a patient with AIDS. *Mount Sinai J Med* **55**, 181-186.

Long, J.A., Jr., Herdt, J.R., Di Chiro, G. and Cramer, H.R. (1980) Cerebral mass lesions in torulosis demonstrated by computed tomography. *J Comp Assist Tomog* **4**, 766-769.

Louria, D.B., Lavenhar, M., Kaminski, T. and Eng, R.H. (1989) Garlic (Allium sativum) in the treatment of experimental cryptococcosis. *J Med Vet Mycol* **27**, 253-256.

Macher, A.M., Bennett, J.E., Gadek, J.E. and Frank, M.M. (1978) Complement depletion in cryptococcal sepsis. *J Immunol* **120**, 1686-1690.

Madrenys, N., De Vroey, C., Raes-Wuytack, C. and Torres-Rodriguez, J.M. (1993) Identification of the perfect state of *Cryptococcus neoformans* from 195 clinical isolates including 84 from AIDS patients. *Mycopathologia* **123**, 65-68.

Manfredi, R., Coronado, O.V., Mastroianni, A. and Chiodo, F. (1997a) Liposomal amphotericin B and recombinant human granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) in the treatment of paediatric AIDS-related cryptococcosis. *Int J STD AIDS* **8**, 406-408.

Manfredi, R., Mastroianni, A., Coronado, O.V. and Chiodo, F. (1997b) Fluconazole as prophylaxis against fungal infection in patients with advanced HIV infection. *Arch Int Med* **157**, 64-69.

McManus, E.J. and Jones, J.M. (1985) Detection of a Trichosporon beigelii antigen cross-reactive with *Cryptococcus neoformans* capsular polysaccharide in serum from a patient with disseminated Trichosporon infection. *J Clin Microbiol* **21**, 681-685.

Medoff, G., Comfort, M. and Kobayashi, G.S. (1971) Synergistic action of amphotericin B and 5-fluorocytosine against yeast-like organisms. *Proc Soc Exp Biol Med* **138**, 571-574.

Menon, A. and Rajamani, R. (1976) Giant 'cryptococcoma' of the lung. *Brit J Dis Chest* **70**, 269-272.

Merchant, J.C. (1983) Agile Wallaby. In: Strahan, R. (Ed.) *The Australian Museum Complete Book of Australian Mammals*, pp. 242-243. Sydney: Angus & Robertson.

Meyer, W., Lieckfeldt, E., Kuhls, K., Freedman, E.Z., Borner, T. and Mitchell, T.G. (1993) DNA- and PCR-fingerprinting in fungi. In: Pena,S.D.J., Chakraoborty,R., Epplen,J.T., and A.J.Jeffreys (Eds.) *DNA fingerprinting: state of the science.* pp. 311-320 Basel: Birkhauser Verlag.

Milchgrub, S., Visconti, E. and Avellini, J. (1990) Granulomatous prostatitis induced by capsule-deficient cryptococcal infection. *J Urol* **143**, 365-366.

Miller, M.F., Mitchell, T.G., Storkus, W.J. and Dawson, J.R. (1990) Human natural killer cells do not inhibit growth of *Cryptococcus neoformans* in the absence of antibody. *Infect Immun* **58**, 639-645.

Miller, M.F. and Mitchell, T.G. (1991) Killing of *Cryptococcus neoformans* strains by human neutrophils and monocytes. *Infect Immun* **59**, 24-28.

Miller, W.T., Jr., Edelman, J.M. and Miller, W.T. (1990) Cryptococcal pulmonary infection in patients with AIDS: radiographic appearance. *Radiology* **175**, 725-728.

Mitchell, D.H., Sorrell, T.C., Allworth, A.M., Heath, C.H., McGregor, A.R., Papanaoum, K., Richards, M.J. and Gottlieb, T. (1995) Cryptococcal disease of the CNS in immunocompetent hosts: influence of cryptococcal variety on clinical manifestations and outcome. *Clin Infect Dis* **20**, 611-616.

Mitchell, T.G. and Perfect, J.R. (1995) Cryptococcosis in the era of AIDS-100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev* **8**, 515-548.

Mody, C.H. and Syme, R.M. (1993) Effect of polysaccharide capsule and methods of preparation on human lymphocyte proliferation in response to *Cryptococcus neoformans*. *Infect Immun* **61**, 464-469.

Montagna, M.T., Tortorano, A.M., Fiore, L., Viviani, M.A. and Barbuti, S. (1996) First isolation of *Cryptococcus neoformans* var. *gattii* in Apulia, Italy. *3rd International Conference on Cryptococcus and Cryptococcosis.* September, Paris 159 (Abstract).

Mukherjee, S., Lee, S.C. and Casadevall, A. (1995) Antibodies to *Cryptococcus neoformans* glucuronoxylomannan enhance antifungal activity of murine macropages. *Infect Immun* **63**, 573-579.

Murphy, J.W., Mosley, R.L., Cherniak, R., Reyes, G.H., Kozel, T.R. and Reiss, E. (1988) Serological, electrophoretic, and biological properties of *Cryptococcus neoformans* antigens. *Infect Immun* **56**, 424-431.

Murphy, J.W. and Cozad, G.C. (1972) Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. *Infect Immun* **5**, 896-901.

National Committee for Clinical Laboratory Standards. (1992) Reference method for broth dilution antifungal susceptibility testing of yeast. Proposed standared M27-P. Villanova, Pa. National Committee for Clinical Laboratory Standards.

Neilson, J.B., Fromtling, R.A. and Bulmer, G.S. (1977) *Cryptococcus neoformans*: size range of infectious particles from aeresolized soil. *Infect Immun* **17,** 634-638.

Newberry, W.M., Jr., Walter, J.E., Chandler, J.W., Jr. and Tosh, F.E. (1967) Epidemiologic study of *Cryptococcus neoformans*. *Ann Int Med* **67**, 724-732.

Okun, E. and Butler, W.T. (1964) Ophthalmologic complications of cryptococcal meningitis. *Arch Ophthalmol* **71,** 52-57.

Pal, M. (1989) *Cryptococcus neoformans* var. *neoformans* and munia birds. *Mycoses* **32**, 250-252.

Pal, M. and Mehrotra, B.S. (1985) Studies on the isolation of *Cryptococcus neoformans* from fruits and vegetables. *Mykosen* **28**, 200-205.

Pathmanathan, R. and Soo-Hoo Tuck Soon (1982) Cryptococcosis in the University Hospital, Kuala Lumpur and review of published cases. *Trans R Soc Trop Med Hyg* **76**, 21-24.

Paugam, A., Dupouy-Camet, J., Blanche, P., Gagneux, J.P., Tourte-Schaeferc, C. and Sicard, D. (1994) Increased fluconazole resistance of *Cryptococcus neoformans* isolated from a patient with AIDS and recurrent meningitis. *Clin Infect Dis* **19,** 975-976.

Pema, K., Diaz, J., Guerra, L.G., Nabhan, D. and Verghese, A. (1994) Disseminated cutaneous cryptococcosis. Comparison of clinical manifestations in the pre-AIDS and AIDS eras. *Arch Int Med* **154**, 1032-1034.

Perfect, J.R., Lang, S.D. and Durack, D.T. (1980) Chronic cryptococcal meningitis: a new experimental model in rabbits. *Am J Pathol* **101**, 177-194.

Perfect, J.R. (1989) Cryptococcosis. *Infect Dis Clin N Amer* **3**, 77-102.

Perfect, J.R., Lindsay, M.H. and Drew, R.H. (1992) Adverse drug reactions to systemic antifungals. *Drug Safety* **7,** 323-363.

Perfect, J.R. and Durack, D.T. (1985) Comparison of amphotericin B and N-D-ornithyl amphotericin B methyl ester in experimental cryptococcal meningitis and Candida albicans endocarditis with pyelonephritis. *Antimicrob Agents Chemother* **28**, 751-755.

Perla, E.N., Maayan, S., Miller, S.N., Ramaswamy, G. and Eisenberg, H. (1985) Disseminated cryptococcosis presenting as the adult respiratory distress syndrome. *N Y State J Med* **85**, 704-706.

Pettoello-Mantovani, M., Casadevall, A., Kollmann, T.R., Rubinstein, A. and Goldstein, H. (1992) Enhancement of HIV-1 infection by the capsular polysaccharide of *Cryptococcus neoformans. Lancet* **339**, 21-23.

Pfeiffer, T. and Ellis, D. (1991) Environmental isolation of *Cryptococcus neoformans gattii* from California. *J Infect Dis* **163**, 929-930.

Pfeiffer, T.J. and Ellis, D. (1997) Additional eucalyptus hosts for *Cryptococcus neoformans* var. *gattii. Abstracts of the 13th Congress of the International Society for Human and Animal Mycology (ISHAM).* North Adelaide, Australia 106 (Abstract).

Pfeiffer, T.J. and Ellis, D.H. (1992) Environmental isolation of *Cryptococcus neoformans* var. *gattii* from Eucalyptus tereticornis. *J Med Vet Mycol* **30**, 407-408.

Pfrommer, G.S., Dickens, S.M., Wilson, M.A., Young, B.J. and Kozel, T.R. (1993) Accelerated decay of C3b to iC3b when C3b is bound to the *Cryptococcus neoformans* capsule. *Infect Immun* **61**, 4360-4366.

Polacheck, I., Lebens, G. and Hicks, J.B. (1992) Development of DNA probes for early diagnosis and epidemiological study of cryptococcosis in AIDS patients. *J Clin Microbiol* **30**, 925-930.

Polacheck, I. and Kwon-Chung, K.J. (1986) Canavanine resistance in *Cryptococcus neoformans. Antimicrob Agents Chemother* **29**, 468-473.

Polak, A. (1978) Synergism of polyene antibiotics with 5-fluorocytosine. *Chemotherapy* **24**, 2-16.

Polak, A., Scholer, H.J. and Wall, M. (1982) Combination therapy of experimental candidiasis, cryptococcosis and aspergillosis in mice. *Chemotherapy* **28**, 461-479.

Poli, G., Bressler, P., Kinter, A., Duh, E., Timmer, W.C., Rabson, A., Justement, J.S., Stanley, S. and Fanci, A.S. (1990) Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumour necrosis factor alpha by transcriptional and post-transcriptional mechanisims. *J Exp Med* **172,** 151-158.

Powderly, W.G., Saag, M.S., Cloud, G.A., Robinson, P., Meyer, R.D., Jacobson, J.M., Graybill, J.R., Sugar, A.M., McAuliffe, V.J., Follansbee, S.E. and et al (1992) A controlled trial of fluconazole or amphotericin B to prevent relapse of cryptococcal meningitis in patients with the acquired immunodeficiency syndrome. The NIAID AIDS Clinical Trials Group and Mycoses Study Group. *N Engl J Med* **326**, 793-798.

Powderly, W.G., Finkelstein, D.M. and Feinberg, J. (1995) A randomized trial comparing fluconazole with clotrimazole troches for the prevention of fungal infections in patients with advanced human immunodeficiency virus infection. *N Engl J Med* **332**, 700-705.

Powell, K.E., Dahl, B.A., Weeks, R.J. and Tosh, F.E. (1972) Airborne *Cryptococcus neoformans*: particles from pigeon excreta compatible with alveolar deposition. *J Infect Dis* **125**, 412-415.

Procknow, J.J., Benfield, J.R., Rippon, J.W., Diener, C.F. and Archer, F.L. (1965) Cryptococcal hepatitis presenting as a surgical emergency. First isolation of *Cryptococcus neoformans* from point source in Chicago. *JAMA* **191**, 269-274.

Pryor, L.D. and Johnson, L.A.S. (1971) *A classification of the eucalypts*, 1st edn. Canberra: Australian National University.

Reiss, F., Szilagyi, G. and Mayer, E. (1975) Immunological studies of the anticryptococcal factor of normal human serum. *Mycopathologia* **55**, 175-178.

Retini, C., Vecchiarelli, A., Monari, C., Tascini, C., Bistoni, F. and Kozel, T.R. (1996) Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. *Infect Immun* **64**, 2897-2903.

Rex, J.H., Larsen, R.A., Dismukes, W.E., Cloud, G.A. and Bennett, J.E. (1993) Catastrophic visual loss due to *Cryptococcus neoformans* meningitis. *Medicine* **72**, 207-224.

Rhodes, J.C., Polacheck, I. and Kwon-Chung, K.J. (1982) Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. *Infect Immun* **36**, 1175-1184.

Richardson, M.D., White, L.J., McKay, I.C. and Shankland, G.S. (1993) Differential binding of acapsulate and encapsulated strains of *Cryptococcus neoformans* to human neutrophils. *J Med Vet Mycol* **31**, 189-199.

Richardson, P.M., Mohandas, A. and Arumugasamy, N. (1976) Cerebral cryptococcosis in Malaysia. *J Neurol Neurosurg Psych* **39**, 330-337.

Riley, E. and Cahan, W.G. (1972) Pulmonary cryptococcoccis followed by pulmonary tuberculosis. A case report. *Am Rev Resp Dis* **106**, 594-599.

Rinaldi, M.G., Drutz, D.J., Howell, A., Sande, M.A., Wofsy, C.B. and Hadley, W.K. (1986) Serotypes of *Cryptococcus neoformans* in patients with AIDS *J Infect Dis* **153**, 642

Rippon, J.W. (1988) *Med. mycology. The pathogenic fungi and the pathogenic actinomycetes.* 3rd edn. pp. 582-609. Philadelphia: WB Saunders.

Ruiz, A. and Bulmer, G.S. (1981) Particle size of airborn *Cryptococcus neoformans* in a tower. *Appl Environ Microbiol* **41**, 1225-1229.

Saag, M.S., Powderly, W.G., Cloud, G.A., Robinson, P., Grieco, M.H., Sharkey, P.K., Thompson, S.E., Sugar, A.M., Tuazon, C.U., Fisher, J.F. and et al (1992) Comparison of amphotericin B with fluconazole in the treatment of acute AIDS-associated cryptococcal meningitis. The NIAID Mycoses Study Group and the AIDS Clinical Trials Group. *N Engl J Med* **326**, 83-89.

Sabetta, J.R. and Andriole, V.T. (1985) Cryptococcal infection of the central nervous system. *Med Clin N Amer* **69**, 333-344.

Sadamoto, S., Ikeda, R., Nishikawa, A. and Shinoda, T. (1993) Evidence for interference by immune complexes in the serodiagnosis of cryptococcosis. *Microbiol Immunol* **37**, 129-133.

Sahu, A., Kozel, T.R. and Pangburn, M.K. (1994) Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation. *Biochem J* **302**, 429-436.

Salas, S.D., Bennett, J.E., Kwon-Chung, K.J., Perfect, J.R. and Williamson, P.R. (1996) Effect of the laccase gene CNLAC1, on virulence of *Cryptococcus neoformans. J Exp Med* **184**, 377-386.

Sanfelice, F. (1894) Contributo alla morfologia e biologia dei blastomiceti che si sviluppano nei succhi di alcuni frutti. *Ann Igien* **4**, 463-495.

Sanfelice, F. (1895) Uber einen neuen Pathogen Blastomyceten welcher innerhalb der Gewebe unter Bildung, Kalkartig aussehender Massendegeneriert. *Zentralbl Bakteriol* **18,** 521-526.

Sarosi, G.A., Parker, J.D., Doto, I.L. and Tosh, F.E. (1969) Amphotericin B in cryptococcal meningitis. Long-term results of treatment. *Ann Int Med* **71**, 1079-1087.

Sarosi, G.A., Silberfarb, P.M. and Tosh, F.E. (1971) Cutaneous cryptococcosis. A sentinel of disseminated disease. *Arch Dermatol* **104**, 1-3.

Sawaya, B.P., Weihprecht, H. and Campbell, W.E. (1991) Direct vasoconstriction as a possible cause of amphotericin B -induced nephrotoxicity in rats. *J Clin Invest* **87,** 2097-2107.

Schimpff, S.C. and Bennett, J.E. (1975) Abnormalities in cell-mediated immunity in patients with *Cryptococcus neoformans* infection. *J All Clin Immunol* **55**, 430-441.

Schupbach, C.W., Wheeler, C.E., Jr., Briggaman, R.A., Warner, N.A. and Kanof, E.P. (1976) Cutaneous manifestations of disseminated cryptococcosis. *Arch Dermatol* **112**, 1734-1740.

Schurmann, D., de Matos Marques, B., Grunewald, T., Pohle, H.D., Hahn, H. and Ruf, B. (1991) Safety and efficacy of liposomal amphotericin B in treating AIDS-associated disseminated cryptococcosis. *J Infect Dis* **164**, 620-622.

Scrimgeour, E.M. and Purohit, R.G. (1984) Chronic pulmonary cryptococcosis in a Rattus rattus from Rabaul, Papua New Guinea. *Trans R Soc Trop Med Hyg* **78**, 827-828.

Seaton, R.A. (1996) Clinical and epidemiological features of cryptococcal meningitis in Papua New Guinea. *Neurol Infect Epidemiol* **1**, 81-90.

Seaton, R.A., Naraqi, S., Wembri, J.P. and Warrell, D.A. (1996) Predictors of outcome in *Cryptococcus neoformans* var. *gattii* meningitis. *Q J Med* **89**, 423-428.

Seaton, R.A., Verma, N., Naraqi, S., Wembri, J.P. and Warrell, D.A. (1997a) The effect of corticosteroids on visual loss in *Cryptococcus neoformans* var. *gattii* meningitis. *Trans R Soc Trop Med Hyg* **91**, 50-52.

Seaton, R.A., Verma, N., Naraqi, S., Wembri, J.P. and Warrell, D.A. (1997b) Visual loss in immunocompetent patients with *Cryptococcus neoformans* var. *gattii* meningitis. *Trans R Soc Trop Med Hyg* **91**, 44-49.

Shadomy, H.J., Wood-Helie, S., Shadomy, S., Dismukes, W.E. and Chau, R.Y. (1987) Biochemical serogrouping of clinical isolates of *Cryptococcus neoformans* [published erratum appears in Diagn Microbiol Infect Dis 1987 Jun;7(2):159]. *Diagnost Microbiol Infec Dis* **6**, 131-138.

Shapiro, L.L. and Beal, J.B. (1925) Torula meningitis. *Arch Neurol Psych* **13,** 174.

Shen, J., Davis, L.E., Wallace, J.M., Cai, Y. and Lawson, L.D. (1996) Enhanced diallyl trisulfide has in vitro synergy with amphotericin B against *Cryptococcus neoformans. Planta Medica* **62**, 415-418.

Similowski, T., Datry, A., Jais, P., Katlama, C., Rosenheim, M. and Gentilini, M. (1989) AIDS-associated Cryptococcosis causing adult respiratory distress syndrome. *Respiratory Medicine* **83**, 513-515.

Skelton, D. (1981) Reforestation in Papua New Guinea. CSIRO Pulp and Paper Seminar. Boroka, PNG: Office of Forests.

Slater, K. (1959) Womersley, J.S. and McAdam, J.B. (Eds.) *The Forests and Forest Conditions in the Territories of Papua and New Guinea.* Port Moresby: W.S.Nicholas

Slobodniuk, R. and Naraqi, S. (1980) Cryptococcal meningitis in the central province of Papua New Guinea. *PNG Med J* **23**, 111-116.

Small, J.M., Mitchell, T.G. and Wheat, R.W. (1986) Strain variation in composition and molecular size of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. *Infect Immun* **54**, 735-741.

Smith, D.K., Neal, J.J. and Holmberg, S.D. (1993) Unexplained opportunistic infections and CD4+ T-lymphocytopenia without HIV infection. An investigation of cases in the United States. *N Engl J Med* **328**, 373-379.

Snedecor, G.W. and Irwin, M.R. (1933) Statistical methods. *Iowa State College of Science* **8,** 75.

Sorrell, T.C., Chen, S.C., Ruma, P., Meyer, W., Pfeiffer, T.J., Ellis, D.H. and Brownlee, A.G. (1996) Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *J Clin Microbiol* **34**, 1253-1260.

Speed, B. and Dunt, D. (1995) Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans. Clin Infect Dis* **21**, 28-34.

Speed, B.R. (1990) The epidemiology of cryptococcosis in Victoria. *Aust N Z J Med* **20**, 435.

Speed, B.R. Update on the clinical differences between the two varieties of *Cryptococcus neoformans*. 120-122. Paris, France: *3rd International Conference on Cryptococcus and Cryptococcosis.* (1996) (Abstract).

Spickard, A., Butler, W.T., Andriole, V. and Utz, J.P. (1963) The improved prognosis of cryptococcal meningitis with amphotericin B therapy. *Ann Int Med* **68**, 66-83.

Spiropulu, C., Eppard, R.A., Otteson, E. and Kozel, T.R. (1989) Antigenic variation within serotypes of *Cryptococcus neoformans* detected by monoclonal antibodies specific for the capsular polysaccharide. *Infect Immun* **57**, 3240-3242.

Spitzer, E.D., Spitzer, S.G., Freundlich, L.F. and Casadevall, A. (1993) Persistence of initial infection in recurrent *Cryptococcus neoformans* meningitis. *Lancet* **341**, 595-596.

Spitzer, E.D. and Spitzer, S.G. (1992) Use of a dispersed repetitive DNA element to distinguish clinical isolates of *Cryptococcus neoformans*. *J Clin Microbiol* **30**, 1094-1097.

Staib, F. (1962a) *Cryptococcus neoformans* beim Kanarienvogel. *Zentralbl Bakteriol* **185,** 129-134.

Staib, F. (1962b) Vogelkot, ein Nashsubtrat fur die Gattung *Cryptococcus*. *Zentralbl Bakteriol* **186,** 233-247.

Staib, F. (1987) Cryptococcosis in AIDS: mycological, diagnostic and epidemiological observations. *Aids-Forschung* **2**, 363-382.

Staib, F., Seibold, M., Antweiler, E., Frohlich, B., Weber, S. and Blisse, A. (1987) The brown colour effect (BCE) of *Cryptococcus neoformans* in the diagnosis, control and epidemiology of C. *neoformans* infections in AIDS patients. *Zentralblat Bakteriol Mikrobiol Hyg* **266**, 167-177.

Staib, F., Seibold, M., Antweiler, E. and Frohlich, B. (1989a) Staib agar supplemented with a triple antibiotic combination for the detection of *Cryptococcus neoformans* in clinical specimens. *Mycoses* **32**, 448-454.

Staib, F., Seibold, M., L'age, M., Heise, W., Skorde, J., Grosse, G., Nurnberger, F. and Bauer, G. (1989b) *Cryptococcus neoformans* in the seminal fluid of an AIDS patient. A contribution to the clinical course of cryptococcosis. *Mycoses* **32**, 171-180.

Staib, F. and Heissenhuber, M. (1989) *Cryptococcus neoformans* in bird droppings: a hygienic-epidemiological challenge. *Aids-Forschung* **12**, 649-655.

Staib, F. and Senska, M. (1973) Brown pigmentation (brown colour effect, BCE) of *Cryptococcus neoformans* on Guizotia abyssinica-creatinine-agar as dependent on the initial pH value. *Zentralbl Bakteriol Parasit Infekt Hyg* **225**, 113-124.

Stamm, A.M., Diasio, T.B., Dismukes, W.E., Shadomy, S., Cloud, G.A., Bowles, C.A., Karam, G.H. and Espinel-Ingroff, A. (1987) Toxicity of amphotericin B plus flucytosine in 194 patients with cryptococcal meningitis. *Am J Med* **83**, 236-242.

Stern, J.J., Hartman, B.J., Sharkey, P., Rowland, V., Squires, K.E., Murray, H.W. and Graybill, J.R. (1988) Oral fluconazole therapy for patients with acquired immunodeficiency syndrome and cryptococcosis: experience with 22 patients. *Am J Med* **85**, 477-480.

Stockman, L. and Roberts, G.D. (1983) Specficity of the latex test for cryptococcal antigen: a rapid, simple method for eliminating factors (corrected version). *J Clin Microbiol* **17,** 945-947.

Stoddard, J.L. and Cutler, E.C. (1916) Torula infection in man. 6, pp.1-98. New York: Rockerfeller Institute for Medical Research Monograph.

Subramanian, S., Kherdekar, S.S., Babu, P.G. and Christianson, C.S. (1982) Lipoid pneumonia with *Cryptococcus neoformans* colonisation. *Thorax* **37**, 319-320.

Sundstrom, J.B. and Cherniak, R. (1993) T-cell-dependent and T-cell-independent mechanisms of tolerance to glucuronoxylomannan of *Cryptococcus neoformans* serotype A. *Infect Immun* **61**, 1340-1345.

Swinne, D., Kayembe, K. and Niyimi, M. (1986a) Isolation of saprophytic *Cryptococcus neoformans* var. *neoformans* in Kinshasa, Zaire. *Ann Soc Belge Med Trop* **66**, 57-61.

Swinne, D., Nkurikiyinfura, J.B. and Muyembe, T.L. (1986b) Clinical isolates of *Cryptococcus neoformans* from Zaire. *Eur J Clin Microbiol* **5**, 50-51.

Swinne, D., Taelman, H., Batungwanayo, J., Bigirankana, A. and Bogaerts, J. (1994) Ecology of *Cryptococcus neoformans* in central Africa *Med Trop* **54**, 53-55.

Swinne, D. and Ed: Torres-Rodriguez, J.M. (1988) Ecology of *Cryptococcus neoformans* and epidemiology of cryptococcosis in the old world. pp.113-119. Barcelona: Prous Scientific.

Swinne-Desgain, D. (1975) *Cryptococcus neoformans* of saprophytic origin. *Sabouraudia* **13**, 303-308.

Swinne-Desgain, D. (1976) *Cryptococcus neoformans* in the crops of pigeons following its experimental administration. *Sabouraudia* **14**, 313-317.

Taelman, H., Clerinx, J., Kagame, A., Batungwanayo, J., Nyirabareja, A. and Bogaerts, J. (1991) Cryptococcosis, another growing burden for central Africa. *Lancet* **338**, 761.

Takeo, K., Tanaka, R., Taguchi, H. and Nishimura, K. (1993) Analysis of ploidy and sexual characteristics of natural isolates of *Cryptococcus neoformans*. *Can J Microbiol* **39**, 958-963.

Tanner, D.C., Weinstein, M.P., Fedorciw, B., Joho, K.L., Thorpe, J.J. and Reller, L.B. (1994) Comparison of commercial kits for detection of cryptococcal antigen. *J Clin Microbiol* **32**, 1680-1684.

Tarrand, J.J., Guillot, C., Wenglar, M., Jackson, J., Lajeunesse, J.D. and Rolston, K.V.I. (1991) Clinical comparison of the resin-containing BACTEC 26 Plus and the Isolator 10 blood culturing systems. *J Clin Microbiol* **29** 2245-2249.

Tay, C.H., Chew, W.L. and Lim, L.C. (1972) Cryptococcal meningitis: its apparent increased incidence in the Far East. *Brain* **95**, 825-832.

Temu, P., John, R. and Naraqi, S. (1987) Cryptococcal meningitis: a disease of the rural population in Papua New Guinea. *23rd Annual Med. Symposium of the Med. Society of Papua New Guinea.* Madang, September (Abstract).

Tjia, T.L., Yeow, Y.K. and Tan, C.B. (1985) Cryptococcal meningitis. *J Neurol Neurosurg Psych* **48**, 853-858.

Todaro-Luck, F., Reiss, E., Cherniak, R. and Kaufman, L. (1989) Characterization of *Cryptococcus neoformans* capsular glucuronoxylomannan polysaccharide with monoclonal antibodies. *Infect Immun* **57**, 3882-3887.

Torres, R., C., Villarreal, M., Robles, P., Aparicia, P. and Cano, P. (1993) Comparative study with two treatments for *Cryptococcus neoformans* meningitis in patients with AIDS: granulocuyte-macrophage colony-stimulating factor (GM-CSF) and amphotericin B vs. amphotericin B alone, preliminary report. *2nd International conference on Cryptococcus and cryptococcosis.* September, Milan 130 (Abstract).

Truelsen, K., Young, T. and Kozel, T.R. (1992) In vivo complement activation and binding of C3 to encapsulated *Cryptococcus neoformans*. *Infect Immun* **60**, 3937-3939.

Turner, S.H., Cherniak, R. and Reiss, E. (1984) Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans*. *Carbohydrate Research* **125**, 343-349.

Turner, S.H., Cherniak, R., Reiss, E. and Kwon-Chung, K.J. (1992) Structural variability in the glucuronoxylomannan of *Cryptococcus neoformans* serotype A isolates determined by 13C NMR spectroscopy. *Carbohydr Res* **233**, 205-218.

Turner, S.H. and Cherniak, R. (1991) Multiplicity in the structure of the glucuronoxylomannan of *Cryptococcus neoformans*. In: Latage, J.P. and Boucias, D. (Eds.) *Fungal cell walls and the immune response.* pp. 123-142. New York: Springer-Verlag.

Tynes, B., Mason, K.N., Jennings, A.E. and Bennett, J.E. (1968) Variant forms of pulmonary cryptococcosis. *Ann Int Med* **69**, 1117-1125.

Utz, J.P., Shadomy, S. and McGehee, K.F. (1972) Flucytosine. *N Engl J Med* **286,** 777-778.

Utz, J.P., Garriques, I.L., Sande, M.A., Warner, J.F., Mandell, G.L., McGehee, R.F., Duma, R.J. and Shadomy, S. (1975) Therapy of cryptococcosis with a combination of flucytosine and amphotericin B. *J Infect Dis* **132**, 368-373.

van der Horst, C.M., Saag, M.S., Cloud, G.A., Hamill, R.J., Graybill, J.R., Sobel, J.D., Johnson, P.C., Tuazon, C.U., Kerkering, T., Moskovitz, B.L., Powderly, W.G. and Dismukes, W.E. (1997) Treatment of cryptococcal meningitis associated with the acquired immunodeficiency syndrome. National Institute of Allergy and Infectious Diseases Mycoses Study Group and AIDS Clinical Trials Group. *N Engl J Med* **337**, 15-21.

Vanbreuseghem, R. and Takashio, M. (1970) An atypical strain of *Cryptococcus neoformans* (San Felice) Vuillemin 1894. II. *Cryptococcus neoformans* var. *gattii* var. nov. *Ann Soc Belge Med Trop* **50**, 695-702.

Varma, A. and Kwon-Chung, K.J. (1989) Restriction fragment polymorphism in mitochondrial DNA of *Cryptococcus neoformans*. *J Gen Microbiol* **135**, 3353-3362.

Varma, A. and Kwon-Chung, K.J. (1992) DNA probe for strain typing of *Cryptococcus neoformans*. *J Clin Microbiol* **30**, 2960-2967.

Vartivarian, S.E., Anaissie, E.J., Cowart, R.E., Sprigg, H.A., Tingler, M.J. and Jacobson, E.S. (1993) Regulation of cryptococcal capsular polysaccharide by iron. *J Infect Dis* **167**, 186-190.

Vecchiarelli, A., Monari, C., Baldelli, F., Pietrella, D., Retini, C., Tascini, C., Francisci, D. and Bistoni, F. (1995a) Beneficial effect of recombinant human granulocyte colony-stimulating factor on fungicidal activity of polymorphonuclear leukocytes from patients with AIDS. *J Infect Dis* **171**, 1448-1454.

Vecchiarelli, A., Retini, C., Pietrella, D., Monari, C., Tascini, C., Beccari, T. and Kozel, T.R. (1995b) Downregulation by cryptococcal polysaccharide of tumor necrosis factor alpha and interleukin-1 beta secretion from human monocytes. *Infect Immun* **63**, 2919-2923.

Vecchiarelli, A., Retini, C., Monari, C., Tascini, C., Bistoni, F. and Kozel, T.R. (1996) Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. *Infect Immun* **64**, 2846-2849.

Vogel, R.A. (1966) The indirect fluorescent antibody test for the detection of antibody in human cryptococcal disease. *J Infect Dis* **116,** 573-580.

von Hanseman, D. (1905) Uber eine bischer nicht beobachte Geiner Krankung durch Hefe. *Verh Dtsch Ges Pathol* 732-751.

Vuillemin, P. (1901) Les blastomycetes pathogenes. *Rev Gen Sci Pures Appl* **12,** 732-751.

Wagner, R.P., Levitz, S.M., Tabuni, A. and Kornfeld, H. (1992) HIV-1 envelope protein (gp120) inhibits the activity of human bronchoalveolar macrophages against *Cryptococcus neoformans*. *Am Rev Resp Dis* **146**, 1434-1438.

Wang, Y. and Casadevall, A. (1994) Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. *Infect Immun* **62**, 3004-3007.

Warren, N.G. and Hazen, K.C. (1995) *Candida, Cryptococcus*, and other yeasts of medical importance. In: Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C. and Yolken, R.H. (Eds.) *Manual of Clincial Microbiology.* Sixth edn. pp. 723-737. Washington, D.C. ASM Press.

Washburn, R.G., Bryant-Varela, B.J., Julian, N.C. and Bennett, J.E. (1991) Differences in *Cryptococcus neoformans* capsular polysaccharide structure influence assembly of alternative complement pathway C3 convertase on fungal surfaces. *Molecular Immunology* **28**, 465-470.

Washburn, R.G. (1996) Role of Natural Killer cells in defense against cryptococcosis. *3rd International Conference on Cryptococcus and Cryptococcosis*. September, Paris, France, 55-56 (Abstract).

Wegener, H.H. and Staib, F. (1983) Fatal cryptococcosis in a bird fancier. A clinical case report on pathology, diagnosis and epidemiology of cryptococcosis. *Zentralbl Bakteriol Mikrobiol Hyg* **256**, 231-238.

Weinroth, S.E. and Tuazon, C.U. (1993) Alopecia associated with fluconazole treatment. *Ann Int Med* **119**, 637.

White, M., Cirrincione, C., Blevins, A. and Armstrong, D. (1992) Cryptococcal meningitis: outcome in patients with AIDS and patients with neoplastic disease. *J Infect Dis* **165**, 960-963.

Whittaker, R.H. (1969) New concepts of kingdoms of organisms. *Science* **163,** 150-160.

Wickes, B.L., Moore, T.D. and Kwon-Chung, K.J. (1994) Comparison of the electrophoretic karyotypes and chromosomal location of ten genes in the two varieties of *Cryptococcus neoformans*. *Microbiology* **140**, 543-550.

Wickes, B.L., Mayorga, M.E., Edman, U. and Edman, J.C. (1996) Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the alpha-mating type. *Proc Natl Acad Sci U SA* **93**, 7327-7331.

Williamson, P.R., Bennett, J.E., Robbins, J. and Schneerson, R. (1993) Vaccination for prevention of cryptococcosis. *2nd International conference on Cryptococcus and cryptococcosis.* September, Milan, Italy 60 (Abstract).

Williamson, P.R. (1994) Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *J Bacteriol* **176**, 656-664.

Wilson, D.E., Bennett, J.E. and Bailey, J.W. (1968) Serologic grouping of *Cryptococcus neoformans*. *Proc Soc Exp Biol Med* **127**, 820-823.

Wilson, M.A. and Kozel, T.R. (1992) Contribution of antibody in normal human serum to early deposition of C3 onto encapsulated and nonencapsulated *Cryptococcus neoformans*. *Infect Immun* **60**, 754-761.

Wolfer, J., Grahn, B. and Kerr, D. (1996) Diagnostic ophthalmology. Idiopathic endophthalmitis with secondary retinal detachment. *Can Vet J* **37**, 377-378.

Wong, B., Perfect, J.R., Beggs, S. and Wright, K.A. (1990) Production of the hexitol D-mannitol by *Cryptococcus neoformans* in vitro and in rabbits with experimental meningitis. *Infect Immun* **58**, 1664-1670.

Yates, F. (1934) Chi squared and small numbers. *J Roy Stat Soc* **S1** 217

Young, B.J. and Kozel, T.R. (1993) Effects of strain variation, serotype, and structural modification on kinetics for activation and binding of C3 to *Cryptococcus neoformans*. *Infect Immun* **61**, 2966-2972.

Yu, Y.L., Lau, Y.N., Woo, E., Wong, K.L. and Tse, B. (1988) Cryptococcal infection of the nervous system. *Q J Med* **66**, 87-96.

Zervos, M. and Meunier, F. (1993) Fluconazole (Difulcan): A review. *Int J Antimicrob Agents* **3,** 147-170.

Zhanel, G.G., Karlowsky, J.A., Harding, G.A., Balko, T.V., Zelenitsky, S.A., Friesen, M., Kabani, A., Turik, M. and Hoban, D.J. (1997) In vitro activity of a new semisynthetic echinocandin, LY-303366, against systemic isolates of Candida species, *Cryptococcus neoformans*, Blastomyces dermatitidis, and Aspergillus species. *Antimicrob Agents Chemother* **41**, 863-865.

Zimmer, B.L. and Roberts, G.D. (1979). Rapid slective urease test for presumptive identification of *Cryptococcus neoformans*. *J Clin Microbiol* **10**, 380-381.

Zimmerman, L.E. and Rappaport, H. (1954) Occurence of cryptococcosis in patients with malignant disease of reticuloendothelial system. *Am J Clin Pathol* **24,** 1050-1072.

Zuger, A., Louie, E., Holzman, R.S., Simberkoff, M.S. and Rahal, J.J. (1986) Cryptococcal disease in patients with the acquired immunodeficiency syndrome. Diagnostic features and outcome of treatment. *Ann Int Med* **104**, 234-240.